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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/EP99/10295</p> <p>(22) International Filing Date: 21 December 1999 (21.12.99)</p> <p>(30) Priority Data: 9900630.6 12 January 1999 (12.01.99) GB</p> <p>(71) Applicant (for all designated States except US): SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): ATKINSON, Gillian, Frances [GB/GB]; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). BOON, Ronald, James [GB/GB]; SmithKline Beecham Consumer Healthcare, St George's Avenue, Weybridge, Surrey KT13 0DE (GB). VANDEPAPELIERE, Pierre, G. [BE/BE]; SmithKline Beecham Biologicals s.a., Rue de l'Institut 89, B-1330 Rixensart (BE). WETTENDORFF, Martina, Anne, Cecile [BE/BE]; SmithKline Beecham Biologicals s.a., Rue de l'Institut 89, B-1330 Rixensart (BE).</p>		<p>(74) Agent: PRIVETT, Kathryn, Louise; SmithKline Beecham Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlessex TW8 9EP (GB).</p> <p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>
<p>(54) Title: NOVEL TREATMENT</p> <p>(57) Abstract</p> <p>This invention provides a pharmaceutical pack comprising as active ingredients (1) an antiviral agent active against hepatitis B virus and (2) a vaccine for the prophylaxis and/or treatment of hepatitis B infection, the active ingredients being for simultaneous or sequential use. Preferred components are a nucleoside analogue as the antiviral agent, together with a hepatitis B virus vaccine which comprises a hepatitis B virus surface antigen.</p>		

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**NOVEL TREATMENT**

This invention relates to the use of a nucleoside analogue active against hepatitis B virus (HBV), or another class of antiviral active against HBV, such as  $\gamma$  interferon or a  
5 nucleotide analogue and an HBV vaccine in the treatment of hepatitis B virus infections.

Chronic hepatitis B virus (HBV) infection, for which there is currently no effective cure, constitutes a global public health problem of enormous dimensions. Chronic  
10 carriers of HBV, estimated to number more than 300 million world-wide, are at risk for development of chronic active hepatitis, cirrhosis and primary hepatocellular carcinoma.

EP-A-388049 (Beecham Group p.l.c.), discloses the use of penciclovir/famciclovir in  
15 the treatment of hepatitis B virus infection. All references herein to penciclovir/famciclovir include pharmaceutically acceptable salts, such as the hydrochloride, and solvates, such as hydrates.

EP-A-494119 (IAF Biochem. International Inc.) discloses the use of 1,3-oxathiolane  
20 nucleoside analogues, including lamivudine, in treatment of Hepatitis B.

The present invention provides a pharmaceutical pack comprising as active ingredients (1) an antiviral agent active against hepatitis B virus and (2) a vaccine for the prophylaxis and/or treatment of hepatitis B infection, the active ingredients being  
25 for simultaneous or sequential use.

By pharmaceutical pack is meant a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredients. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser  
30 device may be accompanied by instructions for administration. Where the antiviral agent and the HBV vaccine are intended for administration as two separate compositions these may be presented in the form of, for example, a twin pack.

The invention may be used for either the treatment or prophylaxis of hepatitis B infections. The invention is most particularly of value for treatment, for example, of  
5 chronic hepatitis B infections.

In one aspect, the antiviral agent as used in the pharmaceutical pack is a nucleoside agent. In a further aspect the antiviral agent is a nucleotide agent. Suitable agents for use in the invention include penciclovir, famciclovir, lamivudine, ganciclovir,  
10 lobucavir, adefovir, ribavirin, BMS200,475, vidarabin or ARA-AMP. Preferred nucleoside analogues include penciclovir, famciclovir and lamivudine.

A further potential antiviral agent is an interferon. Alpha – interferon is especially preferred.

15

Information with respect to structure and activity of nucleoside analogues may be obtained from well known pharmaceutical industry references, such as "Pharmaprojects", PJB publications Limited, Richmond, Surrey, U.K. or from 'R & D Focus', issued by IMS World publications, 364 Euston Road, London NW1 3BL.

20

References to an anti-hepatitis B virus nucleoside analogue, including the specific compounds mentioned hereinbefore and salts thereof, include solvates such as hydrates.

25 Examples of pharmaceutically acceptable salts are as described in the aforementioned Patent reference in the name of Beecham Group p.l.c. and references quoted therein, the subject matter of which are incorporated herein by reference.

It will be appreciated that the anti-hepatitis B virus nucleoside or nucleotide analogue  
30 and HBV vaccine of this invention may be administered in combination with other pharmacologically active agents, in particular, other antivirals.

In this invention the vaccine for the prophylaxis and/or treatment of hepatitis B infection includes all vaccines containing HBV antigens (such as surface antigen, core and polymerase) and therapeutic vaccines.

- 5 In one aspect of the invention the hepatitis B virus antigen is the hepatitis B surface antigen (HbsAg). The preparation of Hepatitis B surface antigen is well documented. See for example, Harford et. al. in Develop. Biol. Standard 54, page 125 (1983), Gregg et. al. in Biotechnology, 5, page 479 (1987), EP-A- 0 226 846, EP-A-0 299 108 and references therein.

10

As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or immunogenic derivative thereof, particularly fragments thereof, displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et. al.

- 15 Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. HBsAg as herein described can also refer to variants, for example the 'escape mutant' described in WO 91/14703. In a further aspect the HBsAg may comprise a protein described as L\* in European Patent Application  
20 Number 0 414 374, that is to say a protein, the amino acid sequence of which consists of parts of the amino acid sequence of the hepatitis B virus large (L) protein (ad or ay subtype), characterised in that the amino acid sequence of the protein consists of either:

- (a) residues 12 - 52, followed by residues 133 - 145, followed by residues  
25 175 - 400 of the said L protein; or  
(b) residue 12, followed by residues 14 - 52, followed by residues 133 - 145, followed by residues 175 - 400 of the said L protein.

HBsAg may also refer to polypeptides described in EP 0 198 474 or EP 0 304 578.

- 30 Normally the HBsAg will be in particle form. It may comprise S protein alone or may be as composite particles, for example (S, L\*) wherein L\* is as defined above and S denotes the S-protein of hepatitis B surface antigen.

A preferred hepatitis B antigen is this composite particle, defined as S,L\*.

5 A further preferred hepatitis B antigen is the 226 amino acid sequence of the HBV surface antigen, in particle form.

Such a vaccine may advantageously include a pharmaceutically acceptable excipient such as a suitable adjuvant. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate (as described in  
10 WO93/24148), but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Advantageously, the hepatitis B virus may be formulated with strong adjuvant  
15 systems. Thus in the formulation of the invention, it is preferred that the adjuvant composition induces an immune response comprising TH1 aspects. Suitable adjuvant systems include, for example a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salts. A vaccine comprising hepatitis B surface antigen in conjunction with 3D-MPL was  
20 described in European Patent Application 0 633 784.

An enhanced system involves the combination of monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in  
25 WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739.

Other known adjuvants which may be included are CpG containing oligonucleotides (see University of Iowa; WO9602555).

30 In a preferred embodiment of the present invention there is provided a vaccine comprising an HBV antigen, adjuvanted with a monophosphoryl lipid A or derivative thereof.

Preferably the vaccine additional comprises a saponin, more preferably QS21.

5 Preferably the formulation additional comprises an oil in water emulsion and tocopherol.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210.

10 The present invention also provides a method of treatment and/or prophylaxis of hepatitis B virus infections, which comprises administering to a human or animal subject, suffering from or susceptible to Hepatitis B virus infection, either either simultaneously or sequentially in any order, a safe and effective amount of 1) an antiviral agent active against hepatitis B virus and 2) a vaccine for the prophylaxis  
15 and/or treatment of hepatitis B infection.

The antiviral such as penciclovir/famciclovir and the HBV vaccine or a pharmaceutically acceptable salt or ester thereof, may be co-administered in the form of two separate pharmaceutical compositions for simultaneous or sequential use. Normally the active ingredients will be administered separately according to the  
20 normal dosage and administration regimen for the ingredients given alone. Commencement of administration may be either with the vaccine or the antiviral.

The present invention also provides for the use of an antiviral compound in the manufacture of a medicament for the treatment of patients already primed with a  
25 hepatitis B vaccine and suffering from a hepatitis B virus infection. The invention further provides for the use of a hepatitis B vaccine in the manufacture of a medicament for the treatment of patients already primed with an antiviral compound and suffering from a hepatitis B virus infection. The preferred antiviral is a nucleoside analogue, most preferably penciclovir/famciclovir or lamivudine.  
30 Preferred hepatitis B vaccines are identified hereinabove.

6

The unit doses of the nucleoside or nucleotide analogue may be administered, for example, 1 to 4 times per day. The exact dose will depend on the route of administration and the severity of the condition being treated, and it will be appreciated that it may be necessary to make routine variations to the dosage depending on the age and weight of the patient and immunocompromised patients may require an increased dosage.

Vaccines are administered in multiple doses at various intervals. This is usually 6 - 12 doses at biweekly or monthly intervals.

10

The preferred ingredients in the pharmaceutical pack when administered simultaneously are given as separate preparations, for example, as vaccinations in each arm. It is however possible to consider simultaneous administration by mixing the ingredients before administration. The ingredients may be given enterally, such as orally or parenterally (e.g. intramuscularly or, more particularly, intravenously).

15

The antiviral agents of the invention may be formulated as a tablet prepared by conventional means. Compositions for oral use such as tablets and capsules may be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, micro-crystalline cellulose or calcium hydrogen phosphate); lubricant (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycollate); or wetting agent (e.g. sodium lauryl sulphate). Tablets may be coated by methods well known in the art.

20

Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); non-aqueous vehicles (e.g. almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives

25

30



(e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled  
5 release of one or both active ingredients.

For parenteral administration the compositions may be presented in a form suitable for bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form e.g. in syringes, ampoules or in multi-dose containers, with an  
10 added preservative.

The active antiviral agent may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredients may be in  
15 powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

For rectal administration the active antiviral agents may be formulated as suppositories or retention enemas, e.g. containing conventional suppository bases  
20 such as cocoa butter or other glycerides.

The active antiviral agents of the invention may be prepared according to conventional techniques well known in the pharmaceutical industry. Thus, for example, the lamivudine/penciclovir/famciclovir may be admixed, if desired, with  
25 suitable excipients. Tablets may be prepared, for example, by direct compression of such a mixture. Capsules may be prepared by filling the blend along with suitable excipients into gelatin capsules, using a suitable filling machine. Controlled release forms for oral or rectal administration may be formulated in a conventional manner associated with controlled release forms.

30

Anti-hepatitis B virus nucleoside analogues may be identified by standard methods, such as tests involving studies in *in vitro* primary duck hepatocyte cultures infected

with the duck hepatitis B virus (DHBV)<sup>8</sup>. Changes in the levels of preS1 and/or viral DNA in cultures treated with such analogs would indicate activity. Alternatively, analogues may be identified by the ability to interfere with normal acylation of synthetic peptides representing the N-terminal amino acids of DHBV or hepatitis B

5   viruses of man, woodchucks, ground squirrels or other animals.

9  
**EXAMPLES**

**Hepatitis B surface antigen vaccine/Lamivudine pharmacokinetics interaction study in dogs**

5

**METHODS**

The following vaccine composition was employed. The HBV surface antigen was equivalent to the antigen employed in the commercially available Engerix-B vaccine

10 <sup>™</sup> (Smithkline Beecham Biologicals), except that it was lyophilised.

**Lyophilized Ag:**

	HBsAg	100µg
	Sucrose	12.6 mg
	NaCl	20.3mM
15	NaH <sub>2</sub> PO <sub>4</sub> / Na <sub>2</sub> HPO <sub>4</sub>	1.35 mM

**Adjuvant system:**

	oil in water emulsion:	250 µl
	- Squalene	10.7 mg
20	- DL α-tocopherol	11.9 mg
	- polyoxyethylenesorbitan monooleate (Tween 80)	4.8 mg
	Monophosphoryl lipid A	100 µg
	QS21	100 µg
25	Water for injection	q.s. ad 0.5 ml
	Na <sub>2</sub> HPO <sub>4</sub>	575 µg
	KH <sub>2</sub> PO <sub>4</sub>	100 µg
	KCl	100 µg
30	NaCl	4mg
	pH	6.8 +/- 0.2

Lamivudine (Zeffix <sup>TM</sup>, GlaxoWellcome) was administered daily by oral capsule to three male and three female dogs at a dose level of 100 mg/dog/day for 6 weeks. On Days 14, 28 and 42 the HBs/adjuvant vaccine as described above was administered by intramuscular injection immediately before administration of Lamivudine. Blood samples were taken at pre-dose, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 hours after dosing of Lamivudine on Days 7, 14, 28 and 42. The separated plasma was frozen at -20°C prior to despatch to Pharma Bio-Research for analysis of plasma concentrations of Lamivudine.

Sera were collected on days 0, 29 and 43 for anti-HBs antibody evaluation.

## RESULTS

### Lamivudine pharmacokinetics

Blood samples were taken on Days 7, 14, 28 and 42 of a 6-week toxicity study in order to assess the systemic exposure of male and female dogs to Lamivudine following daily oral administration of Lamivudine at a dose level of 100 mg/dog/day and intramuscular administration of HBs vaccine on Days 14, 28 and 42 immediately before administration of Lamivudine. Plasma concentrations of Lamivudine in samples taken up to 24 hours post-dose were measured by Pharma Bio-Research.

The maximum mean plasma concentrations of Lamivudine occurred at 2 hours post-dose on all the sampling days except for females on Day 7 where the maximum mean plasma Lamivudine concentration occurred at 1 hour post-dose. On Day 28, the maximum mean plasma concentrations of Lamivudine were lower than those values on Day 7, 14 and 42. After the maximum, the mean plasma concentrations of Lamivudine declined in an apparently biexponential manner.

Mean maximum plasma concentrations (C<sub>max</sub>) of Lamivudine and the areas under the plasma Lamivudine concentration-time curves estimated up to 24

hours post-dose ( $AUC_{24}$ ) on Days 7, 14, 28 and 42 are summarised below with standard deviations in parentheses:

*C<sub>max</sub> (ng/ml)*

5

<i>Day 7</i>		<i>Day 14</i>		<i>Day 28</i>		<i>Day 42</i>	
<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>
3045	4290	3176	3555	2053	2542	3277	3287
(1516)	(3335)	(871)	(1901)	(515)	(1255)	(567)	(1256)

*AUC<sub>24</sub> (ng.h/ml)*

<i>Day 7</i>		<i>Day 14</i>		<i>Day 28</i>		<i>Day 42</i>	
<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>
12541	11514	12858	13567	11629	8883	12585	11049
(2211)	(4324)	(3231)	(5957)	(2694)	(2534)	(1182)	(4334)

- 10 The times at which the maximum plasma concentrations occurred ( $T_{max}$ ) in individual dogs were generally 2 hours, and in the range 0.75 to 4 hours and appeared to be independent of administration of the HBs vaccine.

- Plasma concentrations of Lamivudine were quantifiable in male animal numbers 71 and 73 and in female animal number 70 at all time points on Days 7, 14, 28 and 42, therefore, these animals were continuously exposed to quantifiable concentrations of Lamivudine during a dosing interval.
- 15

- The rate ( $C_{max}$ ) of systemic exposure of female dogs to Lamivudine was slightly higher than that in male dogs. The extent ( $AUC_{24}$ ) of systemic exposure of female dogs to Lamivudine was generally slightly lower than that in male dogs. However, there was no statistically significant evidence for any sex-related differences in systemic exposure ( $p \geq 0.57$ ).
- 20

On Days 14, 28 and 42 the rate ( $C_{max}$ ) and extent ( $AUC_{24}$ ) of systemic exposure of dogs to Lamivudine were generally similar to those values on Day 7, however, the  $C_{max}$  values in female dogs on Days 14, 28 and 42 appeared to be lower than those values on Day 7. Overall, there was no statistically significant evidence for any time (day of sampling) related differences in the rate and extent of systemic exposure ( $p \geq 0.08$ ). The mean values of accumulation ratios, based on  $AUC_{24}$  values are summarised below :

		Accumulation ratio	
		Males	Females
10	Day 14/Day 7	1.0	1.2
	Day 28/Day 7	0.9	0.8
	Day 42/Day 7	1.0	1.0

15 The mean accumulation ratios were generally close to or less than one indicating that little or no accumulation of Lamivudine occurred following administration of HBs vaccine.

The terminal rate constants, and corresponding terminal half-lives, of Lamivudine on Days 7, 14, 28 and 42 are presented in Tables 5 - 8. The terminal rate constant, where it could be calculated ranged from 0.3239 to 0.1364 hours<sup>-1</sup> corresponding to a terminal half-life of Lamivudine of 2.1 to 5.1 hours.

### Serology

25

#### Methodology

Quantitation of anti-HBs antibody was performed by ELISA using HBs (Hep 286) as coating antigen. Antigen and antibody solutions were used at 100  $\mu$ l per well. Antigen was diluted at a final concentration of 1  $\mu$ g/ml in PBS and was adsorbed overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1hr 30 min at 37°C with PBS

containing 5% non fat powder milk and 0.1% Tween 20. Two-fold dilutions of sera (starting at 1/50 or 1/200 dilution) in PBS containing 0.5% Gloria milk and 0.1% Tween 20 were added to the HBs-coated plates and incubated for 1 hr at 37°C. The plates were washed four times with PBS 0.1% Tween 20. HRPO-conjugated anti-dog IgG (Rockland, USA) diluted 1/40000 in 0.5% non fat powder milk and 0.1% Tween 20 buffer was added to each well and incubated for 1 hr at RT. After a washing step, plates were incubated for 10 min at RT with a solution of Tetramethyl benzidine (TMB) (Biorad, USA) 2-fold diluted in Citrate buffer (0.1M pH=5.8). The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> 0.5N and plates were read at 450/630 nm. ELISA titers were expressed as midpoint titers.

### Results

The anti-HBs serologic response was measured by ELISA at day 0, 29 and 43.

Midpoint titers are presented in the following table :

**Midpoint of anti-HBs antibody titers**

Dog #	Day 0	Day 29	Day 43
69	25	679	7258
71	25	389	3780
73	25	705	6496
70	25	63	1027
72	25	176	3821
74	25	582	11482
<b>Average</b>	<b>25</b>	<b>383</b>	<b>5321</b>

20

The mid-point average titers at the different timepoint are the respectively 25 on Day 0 (arbitrary 1/2 of first dilution), 383 on day 29 and 5321 on day 43. This clearly indicate the induction of an immune response.

## CONCLUSION

5 In conclusion, the rate and extent of systemic exposure of dogs to Lamivudine following repeated oral administration of Lamivudine at a dose level of 100 mg/dog/day appeared to be independent of the administration of HBs vaccine on Days 14, 28 and 42 o the 6-week pharmacokinetic interaction study. There was no evidence of a difference in the rate and extent of systemic exposure to Lamivudine between male and female dogs.

10

Administration of the pharmaccine appeared to be immunogenic and induced high circulating levels of anti-HBs antibodies, validating the use of the Beagle dog as an animal species for this PK interaction study.

15



## CLAIMS

1. A pharmaceutical pack comprising as active ingredients (1) an antiviral agent  
5 active against hepatitis B virus and (2) a vaccine for the prophylaxis and/or  
treatment of hepatitis B infection, the active ingredients being for simultaneous or  
sequential use.
2. A pharmaceutical pack as claimed in claim 1 for use in the treatment of hepatitis  
10 B infections.
3. A pharmaceutical pack as claimed in claim 1 for use in the prevention of hepatitis  
B infections.
- 15 4. A pharmaceutical pack as claimed in any one of the preceding claims wherein the  
antiviral agent is a nucleoside analogue.
5. A pharmaceutical pack as claimed in claim 4 wherein the antiviral agent is  
selected from the group comprising; penciclovir, famciclovir or lamivudine.  
20
6. A pharmaceutical pack as claimed in any one of claims 1 – 3 wherein the antiviral  
agent is a nucleotide analogue.
7. A pharmaceutical pack as claimed in claim 4 or claim 6 wherein the antiviral  
25 agent is selected from the group comprising; ganciclovir, lobucavir, adefovir,  
ribavirin, BMS200,475, vidarabin or ARA-AMP.
8. A pharmaceutical pack as claimed in any one of claims 1 – 3 wherein the antiviral  
agent is alpha - interferon.  
30
9. A pharmaceutical pack as claimed in any one of the preceding claims wherein the  
vaccine active against hepatitis B comprises hepatitis B surface antigen.

10. A pharmaceutical pack as claimed in claim 9 wherein the vaccine active against hepatitis B comprises the antigen SL\*.
- 5 11. A pharmaceutical pack as claimed in claim 9 wherein the vaccine active against hepatitis B comprises the 226 amino acid S antigen.
12. A pharmaceutical pack as claimed in any one of the preceding claims in which the vaccine comprises an adjuvant.
- 10 13. A pharmaceutical pack as claimed in claim 12 wherein the adjuvant is selected from the group of adjuvants comprising: 3D-MPL, QS21, a mixture of QS21 and cholesterol, a CpG oligonucleotide, aluminium hydroxide, aluminium phosphate, tocopherol, and an oil in water emulsion or a combination of two or more of the
- 15 said adjuvants.
14. A pharmaceutical pack as claimed in claim 13 wherein the adjuvant comprises 3D-MPL, QS21 and an oil in water emulsion.
- 20 15. A pharmaceutical pack as claimed in claim 14 wherein the oil in water emulsion comprises squalene, tocopherol and polyoxyethylenesorbitan monooleate (Tween 80).
- 25 16. A method of treating a patient suffering from or susceptible to Hepatitis B virus infection, comprising administering to a patient in need thereof, either simultaneously or sequentially in any order, a safe and effective amount of 1) an antiviral agent active against hepatitis B virus and 2) a vaccine for the prophylaxis and/or treatment of hepatitis B infection.
- 30 17. A method as claimed in claim 13 which comprises the use of a pharmaceutical pack according to any of claims 1 to 15

18. Use of an antiviral compound in the manufacture of a medicament for the treatment of patients already primed with a hepatitis B vaccine and suffering from a hepatitis B virus infection.
19. Use of a hepatitis B vaccine in the manufacture of a medicament for the treatment of patients already primed with an antiviral compound and suffering from a hepatitis B virus infection.

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> <b>PCT/EP99/10295</b> <b>(22) International Filing Date:</b> <b>21 December 1999 (21.12.99)</b>  <b>(30) Priority Data:</b> <b>9900630.6</b> <b>12 January 1999 (12.01.99)</b> <b>GB</b>  <b>(71) Applicant (for all designated States except US):</b> <b>SMITHK-</b> <b>LINE BEECHAM BIOLOGICALS S.A. [BE/BE];</b> Rue de <b>l'Institut 89, B-1330 Rixensart (BE).</b>  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> <b>ATKINSON, Gillian,</b> <b>Frances [GB/GB];</b> SmithKline Beecham Pharmaceuticals, <b>New Frontiers Science Park South, Third Avenue, Harlow,</b> <b>Essex CM19 5AW (GB). BOON, Ronald, James [GB/GB];</b> <b>SmithKline Beecham Consumer Healthcare, St George's</b> <b>Avenue, Weybridge, Surrey KT13 0DE (GB). VANDE-</b> <b>PAPELIERRE, Pierre, G. [BE/BE];</b> SmithKline Beecham <b>Biologicals s.a., Rue de l'Institut 89, B-1330 Rixensart</b> <b>(BE). WETTENDORFF, Martine, Anne, Cecile [BE/BE];</b> <b>SmithKline Beecham Biologicals s.a., Rue de l'Institut 89,</b> <b>B-1330 Rixensart (BE).</b>	<b>(74) Agent:</b> <b>PRIVETT, Kathryn, Louise;</b> SmithKline Beecham <b>Corporate Intellectual Property, Two New Horizons Court,</b> <b>Brentford, Middlesex TW8 9EP (GB).</b>  <b>(81) Designated States:</b> <b>AR, AL, AM, AT, AU, AZ, BA, BB, BG,</b> <b>BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE,</b> <b>ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP,</b> <b>KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,</b> <b>MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU,</b> <b>SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US,</b> <b>UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS,</b> <b>MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ,</b> <b>BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE,</b> <b>CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,</b> <b>NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA,</b> <b>GN, GW, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <b>With international search report.</b>  <b>(88) Date of publication of the international search report:</b> <b>9 November 2000 (09.11.00)</b>	
<b>(54) Title:</b> <b>COMBINATION OF HEPATITIS B VACCINE WITH ANTIVIRAL AGENTS</b>  <b>(57) Abstract</b>  <p>This invention provides a pharmaceutical pack comprising as active ingredients (1) an antiviral agent active against hepatitis B virus and (2) a vaccine for the prophylaxis and/or treatment of hepatitis B infection, the active ingredients being for simultaneous or sequential use. Preferred components are a nucleoside analogue as the antiviral agent, together with a hepatitis B virus vaccine which comprises a hepatitis B virus surface antigen.</p>		

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 99/10295

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/29 A61K31/52 A61K31/70 A61K38/21 A61P5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBASE, MEDLINE, EPO-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BONI C ET AL: "Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B 'see comments!'" JOURNAL OF CLINICAL INVESTIGATION, (1998 SEP 1) 102 (5) 968-75., XP000909082 abstract; figures 1,3,4 page 969, column 2, paragraph 6 -page 970 page 973, column 2 — —/—	1-5,9-19

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

21 July 2000

Date of mailing of the international search report

08.08.00

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/10295

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERENGUER M ET AL: "Hepatitis B and C viruses: Molecular identification and targeted antiviral therapies" PROCEEDINGS ASSOCIATION OF AMERICAN PHYSICIANS, vol. 110, no. 2, March 1998 (1998-03), pages 98-112, XP000909240 abstract page 102, column 2	1-5,9-19
X,P	PIANKO S. ET AL: "Chronic hepatitis B: New therapies on the horizon?" LANCET, (13 NOV 1999) 354/9191 (1662-1663)., XP000906960 paragraph '0005!; table 1	1-7,9-19
Y	GROB P J: "Hepatitis B: virus, pathogenesis and treatment" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 1001, November 1998 (1998-11), pages S11-S16, XP004150417 ISSN: 0264-410X page S16, column 2	1-7,9-19
P,X	VALDEZ H ET AL: "Response to immunization with recall and neoantigens after prolonged administration of an HIV-1 protease inhibitor-containing regimen. ACTG 375 team. AIDS Clinical Trials Group." AIDS, (2000 JAN 7) 14 (1) 11-21., XP000909260 see discussion abstract	1-7,9-19
P,X	RUDD, JENNIFER N. (1) ET AL: "Possible role for hepatitis B vaccine after lamivudine rescue for severe acute hepatitis B." GASTROENTEROLOGY, (APRIL, 1999) VOL. 116, NO. 4 PART 2, PP. A1268. MEETING INFO.: DIGESTIVE DISEASE WEEK AND THE 100TH ANNUAL MEETING OF THE AMERICAN GASTROENTEROLOGICAL ASSOCIATION ORLANDO, FLORIDA, USA MAY 16-19, 1999 AMERICAN GASTROENTEROLOGICAL A, XP000909262 abstract	1-5,9-19
-/-		

# INTERNATIONAL SEARCH REPORT

Int. onal Application No  
PCT/EP 99/10295

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 414 374 A (SMITHKLINE BIOLOG) 27 February 1991 (1991-02-27) cited in the application abstract; claims 8,16; example F9	1-7,9-19
P,Y	WO 99 45957 A (SMITHKLINE BEECHAM BIOLOG ;STEPHENNE JEAN (BE); WETTENDORFF MARTIN) 16 September 1999 (1999-09-16) page 5 -page 7	1-7,9-19
X,P	SUK-FONG LOK A.: "Hepatitis B infection: Pathogenesis and management." JOURNAL OF HEPATOLOGY, SUPPLEMENT, (2000) 32/1 (89-97)., XP000909278 abstract page 95	1-7,9-19
X,P	MOLLOY P J ET AL: "Combined interferon, famciclovir and GM-CSF treatment of HBV infection in an individual with periarteritis nodosa." HEPATO-GASTROENTEROLOGY, (1999 JUL-AUG) 46 (28) 2529-31., XP000909277 abstract	1-7,9-19



# INTERNATIONAL SEARCH REPORT

Int. application No.  
PCT/EP 99/10295

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-5,7,9-19  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

As a result of the prior review under R. 40.2(e) PCT,  
no additional fees are to be refunded.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:  
1-3 (partially) 4-6 (complete) 7 (partially) 9-19 (partially)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 99 10295

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-5,7,9-19

Present claims 1-5,7,9-19 relate to a product defined by reference to a desirable characteristic or property, namely "antiviral agent" and "vaccine for the prophylaxis and treatment of hepatitis B infection". The claims cover all products having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Moreover claim 4 relates to an extremely large number of possible compounds defined as "nucleoside analogues". Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed.

Consequently, the search for the first invention has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the products used in the examples and specifically mentioned in claims 5,9-11 with due regard to the general idea underlying the present application.

Claims partially searched: 5, 7 9-11,13-15 as far as relating to the compounds specified in claim 5, the nucleoside analogues specified in claim 7 the vaccine specified in claims 9-11, and the adjuvants specified in claims 13,14,15

Claims not searched: 1-4,12,16-19

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 99 10295

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 4,5 complete, 1-3,7,9-19 partially

Pharmaceutical composition comprising nucleoside analogue and a vaccine for the prophylaxis and/or treatment of hepatitis B infection. Methods of treating hepatitis B virus infection using said combination therapy.

2. Claims: 6 complete, 1-3,7,9-19 partially

Pharmaceutical composition comprising nucleotide analogue and a vaccine for the prophylaxis and/or treatment of hepatitis B infection. Methods of treating hepatitis B virus infection using said combination therapy.

3. Claims: 8 complete, 1-3,7,9-19 partially

Pharmaceutical composition comprising alpha-interferon and a vaccine for the prophylaxis and/or treatment of hepatitis B infection. Methods of treating hepatitis B virus infection using said combination therapy.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/10295

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0414374 A	27-02-1991	AT 159031 T	15-10-1997
		AU 714652 B	06-01-2000
		AU 1896897 A	19-06-1997
		AU 5972890 A	31-01-1991
		AU 7752894 A	16-02-1995
		CA 2021762 A	26-01-1991
		CN 1052331 A	19-06-1991
		DE 69031556 D	13-11-1997
		DE 69031556 T	14-05-1998
		DK 414374 T	09-03-1998
		ES 2109921 T	01-02-1998
		GR 3025819 T	31-03-1998
		HK 1003032 A	30-09-1998
		HU 57266 A	28-11-1991
		JP 3080083 A	04-04-1991
		KR 181940 B	01-04-1999
		NO 304268 B	23-11-1998
		NZ 234615 A	23-12-1992
		PL 168597 B	29-03-1996
		PT 94791 A,B	20-03-1991
		SG 48175 A	17-04-1998
		ZA 9005763 A	28-08-1991
		PL 168408 B	29-02-1996
		PL 168787 B	30-04-1996
WO 9945957 A	16-09-1999	AU 3328399 A	27-09-1999



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>A61K 39/21, 39/39, A61P 31/18</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/67787</b>  <b>(43) International Publication Date:</b> 16 November 2000 (16.11.00)
<b>(21) International Application Number:</b> PCT/US00/12495 <b>(22) International Filing Date:</b> 5 May 2000 (05.05.00)  <b>(30) Priority Data:</b> 60/132,762                      6 May 1999 (06.05.99)                      US 60/150,667                      25 August 1999 (25.08.99)                      US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US    Not furnished (CIP) Filed on    5 May 2000 (05.05.00)  <b>(71) Applicant (for all designated States except US):</b> THE IMMUNE RESPONSE CORPORATION [US/US]; 5935 Darwin Court, Carlsbad, CA 92008 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> MOSS, Ronald, B. [US/US]; 5021 Manor Ridge Lane, San Diego, CA 92130 (US).  <b>(74) Agents:</b> WEBSTER, Melanic, K. et al.; Campbell & Flores LLP, 7th Floor, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> HIV IMMUNOGENIC COMPOSITIONS AND METHODS  <b>(57) Abstract</b>  <p>The invention provides immunogenic compositions which enhance <math>\beta</math>-chemokine levels in a mammal. The immunogenic compositions contain an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. The HIV antigen can be a whole-killed HIV virus devoid of outer envelope protein gp120. Alternatively, the HIV antigen can be a whole-killed HIV virus, or a p24 antigen. Also provided are kits, the components of which, when combined, produce the immunogenic compositions of the invention. The invention also provides methods of making the immunogenic compositions, by combining an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. The invention further provides a method of immunizing a mammal, by enhancing <math>\beta</math>-chemokine production in the mammal by administering to the mammal an immunogenic composition containing an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. Also provided is a method of inhibiting AIDS, by enhancing <math>\beta</math>-chemokine production in the mammal by administering to the mammal an immunogenic composition containing an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant.</p>		

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HIV IMMUNOGENIC COMPOSITIONS AND METHODS

5

BACKGROUND INFORMATION

This invention relates to Acquired  
Immunodeficiency Syndrome (AIDS) and, more specifically,  
10 to immunogenic compositions for use in preventing and  
treating AIDS.

More than 30 million people world wide are now  
infected with the human immunodeficiency virus (HIV), the  
virus responsible for AIDS. About 90% of HIV infected  
15 individuals live in developing countries, including sub-  
Saharan Africa and parts of South-East Asia, although the  
HIV epidemic is rapidly spreading throughout the world.  
Anti-viral therapeutic drugs that reduce viral burden and  
slow the progression to AIDS have recently become  
20 available. However, these drugs are prohibitively  
expensive for use in developing nations. Thus, there  
remains an urgent need to develop effective preventative  
and therapeutic vaccines to curtail the global AIDS  
epidemic.

25 To date, HIV has proven a difficult target for  
effective vaccine development. Because of the propensity  
of HIV to rapidly mutate, there are now numerous strains  
predominating in different parts of the world whose  
epitopes differ. Additionally, in a particular infected  
30 individual, an HIV virus can escape from the control of

the host immune system by developing mutations in an epitope. There remains a need to develop improved HIV vaccines that stimulate the immune system to recognize a broad spectrum of conserved epitopes, including epitopes  
5 from the p24 core antigen.

During the 1990's, more than 30 different candidate HIV-1 vaccines entered human clinical trials. These vaccines elicit various humoral and cellular immune responses, which differ in type and strength depending on  
10 the particular vaccine components. There remains a need to develop HIV vaccine compositions that strongly elicit the particular immune responses correlated with protection against HIV infection.

The nature of protective HIV immune responses  
15 has been addressed through studies of individuals who have remained uninfected despite repeated exposure to HIV, or who have been infected with HIV for many years without developing AIDS. These studies have shown that immune responses of the T helper 1 (Th1) type correlate  
20 well with protection against HIV infection and subsequent disease progression. Besides antigen-specific Th1 responses, CD8+ cytotoxic T cell responses are considered important in preventing initial HIV infection and disease progression. During an effective anti-viral immune  
25 response, activated CD8+ T cells directly kill virus-infected cells and secrete cytokines with antiviral activity.

The  $\beta$ -chemokine system also appears to be important in protection against initial HIV infection and  
30 disease progression. Infection of immune cells by most primary isolates of HIV requires interaction of the virus with CCR5, whose normal biological role is as the



principal receptor for the  $\beta$ -chemokines RANTES, MIP-1 $\alpha$  and MIP- $\beta$ . Genetic polymorphisms resulting in decreased expression of the CCR5 receptor have been shown to provide resistance to HIV infection. Additionally, a  
5 significant correlation between  $\beta$ -chemokine levels and resistance to HIV infection, both in exposed individuals and in cultured cells, has been demonstrated. It has been suggested that  $\beta$ -chemokines may block HIV infectivity by several mechanisms, including competing  
10 with or interfering with HIV binding to CCR5, and downregulating surface CCR5.

Because of the importance of  $\beta$ -chemokines in preventing initial HIV infection and disease progression, an effective HIV immunogenic composition should induce  
15 high levels of  $\beta$ -chemokine production, both prior to infection and in response to infectious virus. However, HIV immunogenic compositions capable of inducing high levels of  $\beta$ -chemokine production have not been described. In particular, immunogenic compositions which stimulate  
20 high levels of  $\beta$ -chemokine production, induce HIV-specific Th1 cellular and humoral immune responses, and induce HIV-specific cytotoxic activity, have not been described.

Compositions that elicit certain types of HIV-specific immune responses may not elicit other important protective responses. For example, Deml et al., Clin. Chem. Lab. Med. 37:199-204 (1999), describes a vaccine containing an HIV-1 gp160 envelope antigen, an immunostimulatory DNA sequence and alum adjuvant, which,  
25 despite inducing an antigen-specific Th1-type cytokine response, was incapable of inducing an antigen-specific cytotoxic T lymphocyte response. Furthermore, a vaccine containing only envelope antigens would not be expected  
30

to induce an immune response against the more highly conserved core proteins of HIV.

Thus, there exists a need for immunogenic compositions and methods that will prevent HIV infection as well as slow progression to AIDS in infected individuals. Ideally, such compositions and methods will elicit potent Th1 cellular and humoral immune responses specific for conserved HIV epitopes, elicit HIV-specific cytotoxic T lymphocyte activity, and stimulate production of high levels of  $\beta$ -chemokines. Such vaccines could be used to prevent maternal transmission of HIV, for vaccination of newborns, children and high-risk individuals, and for vaccination of infected individuals. Such vaccines could also be used in combination with other HIV therapies, including protease inhibitors. The present invention satisfies this need and provides related advantages as well.

#### SUMMARY OF THE INVENTION

The invention provides immunogenic compositions which enhance  $\beta$ -chemokine levels in a mammal. The immunogenic compositions contain an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. The HIV antigen can be a whole-killed HIV virus devoid of outer envelope protein gp120. Alternatively, the HIV antigen can be a whole-killed HIV virus, or a p24 antigen.

In the immunogenic compositions of the invention, the isolated nucleic acid molecule containing an ISS can be an oligodeoxynucleotide. The isolated nucleic acid molecule containing an ISS can contain two

or more CpG sequences. Exemplary ISS-containing nucleic acid molecules contain the motif 5'-Cytosine, Guanine, Pyrimidine, Pyrimidine-3'. The isolated nucleic acid molecule can contain a phosphorothioate backbone. The  
5 isolated nucleic acid molecule can be conjugated to the HIV antigen.

In the immunogenic compositions of the invention, the adjuvant can be suitable for administration to a human. An exemplary adjuvant is  
10 Incomplete Freund's Adjuvant.

The immunogenic compositions of the invention can further enhance HIV-specific IgG2b antibody production in a mammal. The immunogenic compositions of the invention can also enhance an HIV-specific cytotoxic  
15 T lymphocyte response in a mammal.

Also provided are kits, which contain an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. The components of the kits, when combined, produce the  
20 immunogenic compositions of the invention.

The invention also provides methods of making the immunogenic compositions, by combining an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. The  
25 components can be combined *ex vivo* or *in vivo* to arrive at the immunogenic compositions.

The invention also provides a method of immunizing a mammal, by enhancing  $\beta$ -chemokine production in the mammal by administering to the mammal an  
30 immunogenic composition containing an HIV antigen, an

isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. Also provided is a method of inhibiting AIDS, by enhancing  $\beta$ -chemokine production in the mammal by administering to the mammal an immunogenic composition containing an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS) and an adjuvant. In the methods of the invention, the mammal can be a primate, such as a human, or a rodent. In certain embodiments of the method, the primate is a pregnant mother or an infant. A human can be HIV seronegative or HIV seropositive. The immunogenic compositions can advantageously be administered to the mammal two or more times.

15

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A and 1B show control and antigen-stimulated interferon- $\gamma$  (IFN- $\gamma$ ) production for indicated treatment groups.

Figures 2A and 2B show production of total IgG, IgG1 and IgG2 isotypes for indicated treatment groups.

Figures 3A and 3B show control and antigen-stimulated RANTES production for indicated treatment groups.

Figure 4A shows a comparison of IFN- $\gamma$  production following treatment with two different immunostimulatory sequences.

Figure 4B shows a comparison of IFN- $\gamma$  production following treatment with two different immunostimulatory sequences.

Figure 5A shows HIV antigen-stimulated IFN- $\gamma$  production from peripheral blood mononuclear cells.

Figure 5B shows HIV antigen-stimulated IFN- $\gamma$  production from CD4+ cells.

5                Figure 5C shows HIV antigen-stimulated IFN- $\gamma$  production from CD8+ cells.

Figure 6A shows production of total anti-p24 IgG for indicated treatment groups.

10              Figure 6B shows production of anti-p24 IgG1 and IgG2 isotypes for indicated treatment groups.

Figure 7A shows control and antigen-stimulated IFN- $\gamma$  production for different treatment groups.

Figure 7B shows control and antigen-stimulated RANTES production for different treatment groups.

15              Figure 7C shows production of total anti-p24 IgG for different treatment groups.

Figure 7D shows production of anti-p24 IgG1 and IgG2 isotypes for different treatment groups.

20              Figure 7E shows T cell proliferative responses to HIV antigens for different treatment groups.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides immunogenic HIV compositions containing an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory

sequence, and an adjuvant. Also provided are kits containing the components of such compositions, for use together. The invention also provides methods of immunizing a mammal with such compositions, or with the components of such compositions, so as to enhance production of  $\beta$ -chemokines in the immunized mammal. Advantageously, the compositions of the invention can also induce potent Th1 immune responses against a broad spectrum of HIV epitopes, and provide a strong HIV-specific cytotoxic T lymphocyte response. Thus, the immunogenic compositions of the invention are useful for preventing HIV infection and slowing progression to AIDS in infected individuals.

As used herein, the term "HIV" refers to all forms, subtypes and variations of the HIV virus, and is synonymous with the older terms HTLVIII and LAV. Various cell lines permanently infected with the HIV virus have been developed and deposited with the ATCC, including those having accession numbers CCL 214, TIB 161, CRL 1552 and CRL 8543, all of which are described in U.S. Pat. No. 4,725,669 and Gallo, Scientific American 256:46 (1987).

As used herein, the term "whole-killed HIV virus" refers to an intact, inactivated HIV virus.

As used herein, the term "outer envelope protein" refers to that portion of the membrane glycoprotein of a retrovirus which protrudes beyond the membrane, as opposed to the transmembrane protein, gp41.

As used herein, the term "HIV virus devoid of outer envelope proteins" refers to a preparation of HIV particles or HIV gene products devoid of the outer

envelope protein gp120, but contains the more genetically conserved parts of the virus (eg. p24 and gp41).

As used herein, the term "HIV p24 antigen" refers to the gene product of the gag region of HIV, characterized as having an apparent relative molecular weight of about 24,000 daltons designated p24. The term "HIV p24 antigen" also refers to modifications and fragments of p24 having the immunological activity of p24. Those skilled in the art can determine appropriate modifications of p24, such as additions, deletions or substitutions of natural amino acids or amino acid analogs, that serve, for example, to increase its stability or bioavailability or facilitate its purification, without destroying its immunological activity. Likewise, those skilled in the art can determine appropriate fragments of p24 having the immunological activity of p24. An immunologically active fragment of p24 can have from 6 residues from the polypeptide up to the full length polypeptide minus one amino acid.

As used herein, the term "immunostimulatory sequence" or "ISS" refers to a nucleotide sequence containing an unmethylated CpG motif that is capable of enhancing the immune response in a mammal when administered in combination with an antigen. Immunostimulatory sequences are described, for example, in PCT publication WO 98/55495.

As ISS can contain, for example, at least one sequence consisting of 5'-Cytosine, Guanine, Pyrimidine, Pyrimidine-3'. For example, the sequence 5'-CGTT-3' is found in two copies in the sequence designated SEQ ID NO:1, described in Example I, and one copy each of the

sequence 5'-CGTT-3' and the sequence 5'-CGCT-3' are found in the sequence designated SEQ ID NO:4, described in Example IV.

An ISS can contain the hexameric motif 5'-  
5 Purine, Purine, Cytosine, Guanine, Pyrimidine,  
Pyrimidine-3', such as the motif 5'-GACGTT-3', two copies  
of which are found in the nucleotide sequence designated  
SEQ ID NO:1. An ISS can also contain, for example,  
either the octameric motif 5'-Purine, Purine, Cytosine,  
10 Guanine, Pyrimidine, Pyrimidine, Cytosine, Cytosine-3' or  
5'-Purine, Purine, Cytosine, Guanine, Pyrimidine,  
Pyrimidine, Cytosine, Guanine-3', such as the sequence  
5'-AACGTTCG-3'. An exemplary isolated nucleic acid  
molecule containing the ISS motif 5'-AACGTTCG -3' has the  
15 nucleotide sequence designated SEQ ID NO:2, as described  
in Example I.

An ISS can contain more than one unmethylated  
CpG motif, such as two or more CpG motifs. An exemplary  
isolated nucleic acid molecule containing two CpG motifs  
20 has the nucleotide sequence designated SEQ ID NO:1 or the  
sequence designated SEQ ID NO:2, described in Example I,  
below. An exemplary isolated nucleic acid molecule  
containing three unmethylated CpG motifs has the  
nucleotide sequence designated SEQ ID NO:4, as described  
25 in Example IV. SEQ ID NO:4 also contains two copies of  
the hexameric motif 5'-Purine, Pyrimidine, Cytosine,  
Guanine, Pyrimidine, Pyrimidine-3', namely both the  
sequence 5'-GTCGCT-3' and the sequence 5'-GTCGTT-3'.

As used herein, the term "nucleic acid molecule  
30 containing an ISS" refers to a linear, circular or  
branched single- or double-stranded DNA or RNA nucleic  
acid that contains an immunostimulatory sequence. The



term "isolated," with reference to a nucleic acid molecule containing an ISS, is intended to distinguish the ISS-containing nucleic acid molecule from an ISS that may naturally be present in a whole-killed HIV virus preparation. A nucleic acid molecule containing an ISS can contain multiple ISSs. The ISSs can be adjacent within the nucleic acid molecule, or they can be separated by additional nucleotide bases within the nucleic acid molecule. Such a nucleic acid molecule can be of any length greater than 6 bases or base pairs, and is preferably greater than about 15 bases or base pairs, such as greater than about 20 bases or base pairs, and can be several kb in length.

A nucleic acid molecule containing an ISS can be, for example, a synthetic oligonucleotide, a naturally occurring nucleic acid molecule of any species, or a vector. A nucleic acid molecule containing an ISS can contain either natural or modified nucleotides or natural or unnatural nucleotide linkages. Modifications known in the art, include, for example, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. An unnatural nucleotide linkage can be, for example, a phosphorothioate linkage in place of a phosphodiester linkage, which increases the resistance of the nucleic acid molecule to nuclease degradation. Various modifications and linkages are described, for example, in PCT publication WO 98/55495.

As used herein, the term "adjuvant" refers to a substance which, when added to an immunogenic agent, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture. Adjuvants can include, for example,

oil-in-water emulsions, water-in oil emulsions, alum  
(aluminum salts), liposomes and microparticles, such as  
polysytrene, starch, polyphosphazene and  
polylactide/polyglycosides. Adjuvants can also include,  
5 for example, squalene mixtures (SAF-I), muramyl peptide,  
saponin derivatives, mycobacterium cell wall  
preparations, monophosphoryl lipid A, mycolic acid  
derivatives, nonionic block copolymer surfactants, Quil  
A, cholera toxin B subunit, polyphosphazene and  
10 derivatives, and immunostimulating complexes (ISCOMs)  
such as those described by Takahashi et al. (1990) Nature  
344:873-875. For veterinary use and for production of  
antibodies in animals, mitogenic components of Freund's  
adjuvant (both complete and incomplete) can be used. In  
15 humans, Incomplete Freund's Adjuvant (IFA) is a preferred  
adjuvant. Various appropriate adjuvants are well known  
in the art and are reviewed, for example, by Warren and  
Chedid, CRC Critical Reviews in Immunology 8:83 (1988).

As used herein, "AIDS" refers to the  
20 symptomatic phase of HIV infection, and includes both  
Acquired Immune Deficiency Syndrome (commonly known as  
AIDS) and "ARC," or AIDS-Related Complex, as described by  
Adler, Brit. Med. J. 294: 1145 (1987). The immunological  
and clinical manifestations of AIDS are well known in the  
25 art and include, for example, opportunistic infections  
and cancers resulting from immune deficiency.

As used herein, the term "inhibiting AIDS"  
refers to a beneficial prophylactic or therapeutic effect  
of the immunogenic composition in relation to HIV  
30 infection or AIDS symptoms. Such beneficial effects  
include, for example, preventing initial infection of an  
individual exposed to HIV; reducing viral burden in an  
individual infected with HIV; prolonging the asymptomatic

phase of HIV infection; increasing overall health or quality of life in an individual with AIDS; and prolonging life expectancy of an individual with AIDS. A clinician can compare the effect of immunization with the patient's condition prior to treatment, or with the expected condition of an untreated patient, to determine whether the treatment is effective in inhibiting AIDS.

As used herein, the term " $\beta$ -chemokine" refers to a member of a class of small, chemoattractive polypeptides that includes RANTES, macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ) and macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ). The physical and functional properties of  $\beta$ -chemokines are well known in the art.

As used herein, the term "enhances," with respect to an immune response such as  $\beta$ -chemokine production, IgG2b production or cytotoxic T lymphocyte activity, is intended to mean that the immunogenic composition elicits a greater immune response than does a composition containing any two of the three components of the immunogenic composition, administered in the same amounts and following the same immunization schedule. As disclosed herein, the components of the immunogenic compositions of the invention can act in synergy. For example, the immunogenic compositions of the invention can enhance  $\beta$ -chemokine production by eliciting production of a higher concentration of  $\beta$ -chemokine than would be expected by adding the effects of pairwise combinations of components of the immunogenic composition.

The  $\beta$ -chemokine production that is enhanced can be either "HIV-specific  $\beta$ -chemokine production," which

refers to production of a  $\beta$ -chemokine in response to stimulation of T cells with an HIV antigen.

Alternatively, or additionally, the  $\beta$ -chemokine production that is enhanced can be "non-specific  $\beta$ -chemokine production," which refers to production of a  $\beta$ -chemokine in the absence of stimulation of T cells with an HIV antigen.

As used herein, the term "kit" refers to components packaged or marked for use together. For example, a kit can contain an HIV antigen, an ISS and an adjuvant in three separate containers. Alternatively, a kit can contain any two components in one container, and a third component and any additional components in one or more separate containers. Optionally, a kit further contains instructions for combining the components so as to formulate an immunogenic composition suitable for administration to a mammal.

The invention provides an immunogenic composition containing an HIV antigen, a nucleic acid molecule containing an immunostimulatory sequence (ISS), and an adjuvant. The immunogenic composition enhances  $\beta$ -chemokine production in a mammal administered the composition.

In one embodiment, the HIV antigen in the immunogenic composition is a whole-killed HIV virus, which can be prepared by methods known in the art. For example, HIV virus can be prepared by culture from a specimen of peripheral blood of infected individuals. In an exemplary method of culturing HIV virus, mononuclear cells from peripheral blood (e.g. lymphocytes) can be obtained by layering a specimen of heparinized venous blood over a Ficoll-Hypaque density gradient and

centrifuging the specimen. The mononuclear cells are then collected, activated, as with phytohemagglutinin for two to three days, and cultured in an appropriate medium, preferably supplemented with interleukin 2. The virus  
5 can be detected either by an assay for reverse transcriptase, by an antigen capture assay for p24, by immunofluorescence or by electron microscopy to detect the presence of viral particles in cells, all of which are methods well-known to those skilled in the art.

10                   Methods for isolating whole-killed HIV particles are described, for example, in Richieri et al., Vaccine 16:119-129 (1998), and U.S. Patent Nos. 5,661,023 and 5,256,767. In one embodiment, the HIV virus is an HZ321 isolate from an individual infected in Zaire in  
15 1976, which is described in Choi et al., AIDS Res. Hum. Retroviruses 13:357-361 (1997).

                  Various methods are known in the art for rendering a virus non-infectious (see, for example  
20 Hanson, MEDICAL VIROLOGY II (1983), de la Maza and Peterson, eds., Elsevier,). For example, the virus can be inactivated by treatment with chemicals or by physical conditions such as heat or irradiation. Preferably, the virus is treated with an agent or agents that maintain  
25 the immunogenic properties of the virus. For example, the virus can be treated with beta-propiolactone or gamma radiation, or both beta-propiolactone and gamma radiation, at dosages and for times sufficient to inactivate the virus.

30                   In another embodiment, the HIV antigen in the immunogenic composition is a whole-killed HIV virus devoid of outer envelope proteins, which can be prepared by methods known in the art. In order to prepare whole-

killed virus devoid of outer envelope proteins, the isolated virus is treated so as to remove the outer envelope proteins. Such removal is preferably accomplished by repeated freezing and thawing of the virus in conjunction with physical methods which cause the swelling and contraction of the viral particles, although other physical or non-physical methods, such as sonication, can also be employed alone or in combination.

In yet another embodiment, the HIV antigen in the immunogenic composition is a substantially purified gene product of HIV. Such gene products include those products encoded by the gag genes (p55, p39, p24, p17 and p15), the pol genes (p66/p51 and p31-34) and the transmembrane glycoprotein gp41. These gene products may be used alone or in combination with other HIV antigens.

The substantially purified gene product of HIV can be a substantially purified HIV p24 antigen. p24 can be substantially purified from the virus by biochemical methods known in the art, or can be produced by cloning and expressing the appropriate gene in a host organism such as bacterial, fungal or mammalian cells, by methods well known in the art. Alternatively, p24 antigen, or a modification or fragment thereof that retains the immunological activity of p24, can be synthesized, using methods well known in the art, such as automated peptide synthesis. Determination of whether a modification or fragment of p24 retains the immunological activity of p24 can be made, for example, by immunizing a mammal and comparing the immune responses so generated, or testing the ability of the modification or fragment to compete with p24 for binding to a p24 antibody.

The immunogenic compositions of the invention also contain an isolated nucleic acid molecule having at least one immunostimulatory sequence (ISS). The HIV antigen and the nucleic acid molecule can be mixed  
5 together, or can be conjugated by either a covalent or non-covalent linkage. Methods of conjugating antigens and nucleic acid molecules are known in the art, and exemplary methods are described in PCT publication WO 98/55495.

10 A nucleic acid molecule containing an ISS can be prepared using methods well known in the art including, for example, oligonucleotide synthesis, PCR, enzymatic or chemical degradation of larger nucleic acid molecules, and conventional polynucleotide isolation  
15 procedures. Methods of producing a nucleic acid molecule containing an ISS, including a nucleic acid molecule containing one or more modified bases or linkages, are described, for example, in PCT publication WO 98/55495.

Those skilled in the art can readily determine  
20 whether a particular nucleic acid molecule containing an ISS is effective in enhancing a desired immune response in a particular mammal by immunizing a mammal of the same species, or a species known in the art to exhibit similar immune responses, with a composition containing a  
25 particular ISS. For example, an optimal ISS to include in an immunogenic composition for administration to a human can be determined in either a human or a non-human primate, such as a baboon, chimpanzee, macaque or monkey.

The immunogenic compositions of the invention  
30 further contain an adjuvant, such as an adjuvant demonstrated to be safe in humans. An exemplary adjuvant is Incomplete Freund's Adjuvant (IFA). Another exemplary

adjuvant contains mycobacterium cell wall components and monophosphoryl lipid A, such as the commercially available adjuvant DETOX™. Another exemplary adjuvant is alum. The preparation and formulation of adjuvants in  
5 immunogenic compositions are well known in the art.

Optionally, the immunogenic compositions of the invention can contain or be formulated together with other pharmaceutically acceptable ingredients, including sterile water or physiologically buffered saline. A  
10 pharmaceutically acceptable ingredient can be any compound that acts, for example, to stabilize, solubilize, emulsify, buffer or maintain sterility of the immunogenic composition, which is compatible with administration to a mammal and does not render the  
15 immunogenic composition ineffective for its intended purpose. Such ingredients and their uses are well known in the art.

The invention also provides kits containing an HIV antigen, an isolated nucleic acid molecule containing  
20 an ISS, and an adjuvant. The components of the kit, when combined, produce an immunogenic composition which enhances  $\beta$ -chemokine levels in a mammal.

The components of the kit can be combined ex vivo to produce an immunogenic composition containing an  
25 HIV antigen, a nucleic acid molecule containing an ISS and an adjuvant. Alternatively, any two components can be combined ex vivo, and administered with a third component, such that an immunogenic composition forms in vivo. For example, an HIV antigen can be emulsified in,  
30 dissolved in, mixed with, or adsorbed to an adjuvant and injected into a mammal, preceded or followed by injection of the nucleic acid molecule containing the ISS.



Likewise, each component of the kit can be administered separately. Those skilled in the art understand that there are various methods of combining and administering an HIV antigen, an isolated nucleic acid molecule  
5 containing an ISS, and an adjuvant, so as to enhance  $\beta$ -chemokine production in a mammal.

An immunogenic composition of the invention is effective in enhancing  $\beta$ -chemokine production in a mammal administered the composition. As described in Examples I  
10 and III, below, production of the  $\beta$ -chemokine RANTES can be detected and quantitated using an ELISA assay of supernatants of T cells (such as lymph nodes cells or peripheral blood cells) from mammals administered the composition. In order to determine antigen-specific  $\beta$ -  
15 chemokine production, T cells from an immunized mammal can be stimulated with HIV antigen in combination with antigen-presenting thymocytes, and the  $\beta$ -chemokine levels measured in the supernatant. In order to determine non-specific  $\beta$ -chemokine production, either T cell  
20 supernatant or a blood or plasma sample from an immunized mammal can be assayed. Similarly, production of other  $\beta$ -chemokines, such as MIP-1 $\alpha$  and MIP-1 $\beta$ , can be detected and quantitated using commercially available ELISA assays, according to manufacturer's instructions.

25 An immunogenic composition of the invention can further be capable of enhancing HIV-specific IgG2b antibody production in a mammal administered the composition. As described in Examples II and III, below, HIV in combination with ISS, or with IFA, stimulate HIV-  
30 specific IgG1 antibody production, but not HIV-specific IgG2b antibody production. In contrast, the immunogenic compositions of the invention can stimulate potent HIV-specific IgG2b antibody production. High levels of IgG2b

antibodies, which are associated with a Th1 type response, are correlated with protection against HIV infection and progression to AIDS.

An immunogenic composition of the invention can further be capable of enhancing HIV-specific cytotoxic T lymphocyte (CTL) responses in a mammal administered the composition. As described in Example II, below, an HIV antigen in combination with an adjuvant elicited low levels of IFN- $\gamma$  production by either CD4+ T cells or CD8+ T cells. However, when an ISS was included in the composition together with an HIV and an adjuvant, there was a dose-dependent increase in IFN- $\gamma$  production by both CD4+ T cells and CD8+ T cells.

IFN- $\gamma$  production by CD4+ T cells is characterized as a classic Th1-type response. IFN- $\gamma$  production by CD8+ T cells, however, is considered to be a cytotoxic T lymphocyte (CTL) response, and is highly correlated with cytolytic activity. CTL activity is an important component of an effective prophylactic or therapeutic anti-HIV immune response. Methods of determining whether a CTL response is enhanced following administration of an immunogenic composition of the invention are well known in the art, and include cytolytic assays (described, for example, in Deml et al. supra (1999)), and ELISA and ELISPOT assays for CD8-specific IFN- $\gamma$  production (see Examples I and II, below).

The invention also provides a method of immunizing an individual. The method consists of enhancing  $\beta$ -chemokine production in an individual by administering to a mammal an immunogenic composition containing an HIV antigen, an isolated nucleic acid molecule containing an ISS, and an adjuvant. The

components of the immunogenic composition can be administered in any order or combination, such that the immunogenic composition is formed *ex vivo* or *in vivo*.

Preferably, the HIV antigen, ISS and adjuvant  
5 are administered simultaneously or at about the same time, in about the same site. However, administering the components within several minutes or several hours of each other can also be effective in providing an immunogenic composition that enhances  $\beta$ -chemokine  
10 production. Additionally, administering the components at different sites in the mammal can also be effective in providing an immunogenic composition that enhances  $\beta$ -chemokine production.

The immunogenic compositions of the invention  
15 can be administered to a human to inhibit AIDS, such as by preventing initial infection of an individual exposed to HIV, reducing viral burden in an individual infected with HIV, prolonging the asymptomatic phase of HIV infection, increasing overall health or quality of life  
20 in an individual with AIDS, or prolonging life expectancy of an individual with AIDS. As described in Examples I-III, below, administration to a mammal of an immunogenic composition containing an HIV antigen, an isolated nucleic acid molecule containing an immunostimulatory  
25 sequence, and an adjuvant stimulates immune responses correlated with protection against HIV infection and progression to AIDS.

In particular, the immunogenic compositions enhance  $\beta$ -chemokine production more effectively than  
30 would be expected by combination of any two components of the immunogenic compositions. Additionally, the immunogenic compositions promote strong Th1 type immune

responses, including both Th1 type cytokines (e.g. IFN- $\gamma$ ) and Th1 type antibody isotypes (e.g. IgG2b). Thus, the immunogenic compositions of the invention will be effective as vaccines to prevent HIV infection when  
5 administered to seronegative individuals, and to reduce viral burden, prolong the asymptomatic phase of infection, and positively affect the health or lifespan of a seropositive individual.

Individuals who have been exposed to the HIV  
10 virus usually express in their serum certain antibodies specific for HIV. Such individuals are termed "seropositive" for HIV, in contrast to individuals who are "seronegative." The presence of HIV specific antibodies can be determined by commercially available  
15 assay systems.

At the present time, serological tests to detect the presence of antibodies to the virus are the most widely used method of determining infection. Such methods can, however, result in both false negatives, as  
20 where an individual has contracted the virus but not yet mounted an immune response, and in false positives, as where a fetus may acquire the antibodies, but not the virus from the mother. Where serological tests provide an indication of infection, it may be necessary to  
25 consider all those who test seropositive as in fact, being infected. Further, certain of those individuals who are found to be seronegative may in fact be treated as being infected if certain other indications of infection, such as contact with a known carrier, are  
30 satisfied.

The immunogenic compositions of the invention can be administered to an individual who is HIV

seronegative or seropositive. In a seropositive individual, it may be desirable to administer the composition as part of a treatment regimen that includes treatment with anti-viral agents, such as protease inhibitors. Anti-viral agents and their uses in treatment regimens are well known in the art, and an appropriate regimen for a particular individual can be determined by a skilled clinician.

As shown in Example IV, below, administration of the immunogenic compositions of the invention to a primate fetus or to a primate neonate resulted in the generation of a strong anti-HIV immune response, indicating that the immune systems of fetuses and infants are capable of mounting an immune response to such compositions which should protect the child from HIV infection or progression to AIDS. Accordingly, the immunogenic compositions of the invention can be administered to an HIV-infected pregnant mother to prevent HIV transmission to the fetus, or to a fetus, an infant, a child or an adult as either a prophylactic or therapeutic vaccine.

The dose of the immunogenic composition, or components thereof, to be administered in the methods of the invention is selected so as to be effective in stimulating the desired immune responses. Generally, an immunogenic composition formulated for a single administration contains between about 1 to 200  $\mu$ g of protein. Preferably, an immunogenic composition contains about 100  $\mu$ g of protein for administration to a primate, such as a human. As shown in Example IV, below, about 100  $\mu$ g of HIV antigen in an immunogenic composition elicits a strong immune response in a primate. As shown

in Examples I-III, below, about 10 µg of HIV antigen is suitable for administration to a rodent.

The immunogenic composition can further contain from about 0.1 µg/ml to about 1 mg/ml of an isolated  
5 nucleic acid molecule containing an ISS sequence, such as about 1 µg/ml, about 10 µg/ml, or about 100 µg/ml. As shown in Example I, below, a ratio of at least 5:1 by weight of nucleic acid molecule to HIV antigen was more effective than lower ratios for eliciting immune  
10 responses. In rodents, an effective amount of an oligonucleotide containing an ISS in an immunogenic composition is from 5 µg to greater than 50 µg, such as about 100 µg. In primates, about 500 µg of an oligonucleotide containing an ISS is suitable in an  
15 immunogenic composition. Those skilled in the art can readily determine an appropriate amount of ISS to elicit a desired immune response.

As with all immunogenic compositions, the immunologically effective amounts of the components must  
20 be determined empirically, but can be based, for example, on immunologically effective amounts in animal models, such as rodents and non-human primates. Factors to be considered include the antigenicity, the formulation (e.g. volume, type of adjuvant), the route of  
25 administration, the number of immunizing doses to be administered, the physical condition, weight and age of the individual, and the like. Such factors are well known in the vaccine art and it is well within the skill of immunologists to make such determinations without  
30 undue experimentation.

The immunogenic compositions of the invention can be administered locally or systemically by any method

known in the art, including, but not limited to, intramuscular, intradermal, intravenous, subcutaneous, intraperitoneal, intranasal, oral or other mucosal routes. The immunogenic compositions can be administered  
5 in a suitable, nontoxic pharmaceutical carrier, or can be formulated in microcapsules or as a sustained release implant. The immunogenic compositions of the invention can be administered multiple times, if desired, in order to sustain the desired immune response. The appropriate  
10 route, formulation and immunization schedule can be determined by those skilled in the art.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within  
15 the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

#### EXAMPLE I

##### Elicitation of cytokine, antibody and chemokine 20 responses by HIV immunogenic compositions

This example shows that immunogenic compositions containing an HIV antigen, an immunostimulatory nucleic acid molecule and an adjuvant, are potent stimulators of IFN- $\gamma$  production (a Th1  
25 cytokine), antibody responses and  $\beta$ -chemokine production in a mammal. In particular,  $\beta$ -chemokine production is enhanced to a greater extent than would be expected from the additive effects of any two components in the composition. Therefore, immunogenic compositions  
30 containing an HIV antigen, an immunostimulatory nucleic acid molecule and an adjuvant mediate potent immune responses of the types that are important in protecting

against HIV infection and disease progression, indicating that these compositions will be effective prophylactic and therapeutic vaccines.

### Materials and Methods

5 *Oligodeoxynucleotides.* ODN (oligodeoxynucleotides) used in this study were purchased from Retrogen (San Diego, California). They were phosphorothioate-modified to increase resistance to nuclease degradation. The ODN sequences with the corresponding CpG or non-CpG motifs  
10 are underlined in Table 1.

Table 1

ODN	Sequence	Motif	SEQ ID
1826	5' TCCATGACGTTCTGACGTT 3'	CpG	1
Oct	5' TGACTGTGAACGTTCCGAGATGA 3'	CpG	2
15 1745	5' TCCAATGAGCTTCCTGAGTCT 3'	non-CpG	3

*Immunizations.* The HIV-1 antigen was prepared from virus particles obtained from cultures of a chronically infected Hut 78 with a Zairian virus isolate (HZ321) which has been characterized as subtype "M," containing  
20 an *env* A/*gag* G recombinant virus (Choi et al., supra (1997)). The gp120 was depleted during the two-step purification process. The antigen was inactivated by the addition of  $\beta$ -propiolactone and gamma irradiation at 50 kGy. Western blot and HPLC analysis showed undetectable  
25 levels of gp120 in the preparation of this antigen (Prior et al., Pharm. Tech. 19:30-52 (1995)). For in vitro experiments, native p24 was preferentially lysed from purified HIV-1 antigen with 2% triton X-100 and then purified with Pharmacia Sepharose Fast Flow S resin.  
30 Chromatography was carried out at pH = 5.0 and p24 was



eluted with linear salt gradient. Purity of the final product was estimated to be >99% by both SDS (sodium dodecyl sulfate) electrophoresis and reverse phase high pressure liquid chromatography. The ODN was added to the  
5 diluted HIV-1 antigen in a volume of at least 5% of the final volume.

CFA (complete Freund's adjuvant) was prepared by resuspending *mycobacterium tuberculosis* H37RA (DIFCO, Detroit, Michigan) at 10 mg/ml in IFA (DIFCO, Detroit,  
10 Michigan). IFA or ISA 51® was formulated by adding one part of the surfactant Montanide 80 (high purity mannide monoleate, Seppie, Paris) to nine parts of Drakeol 6 VR light mineral oil (Panreco, Karnes City, Pennsylvania). The gp120-depleted HIV-1 antigen was diluted in PBS to  
15 200µg/ml and emulsified with equal volumes of CFA or IFA with or without ODN.

Eight to twelve weeks old Lewis rats from Charles Rivers (Wilmington, Massachusetts), maintained in a pathogen-free facility, were injected intradermally in  
20 the hind footpad with 100µl of emulsion. Each animal received 10µg of the inactivated HIV-1 antigen in either CFA (n=6), IFA (n=6), 50 µg ISS (n=3), or IFA plus 50 µg ISS (n=6). Two weeks later, the animals were boosted subcutaneously in the base of the tail using the same  
25 regimen, except that the animals primed with HIV-1 antigen in CFA were instead boosted with HIV-1 antigen in IFA. Rats were primed and boosted with HIV-1 antigen in the presence of the ODN 1826, which contains an ISS, or ODN 1745, which does not contain an ISS. On day 28, the  
30 animals were sacrificed for cytokine, chemokine, and antibody analysis. For ISS dose response studies, n=3 for all groups.

*ELISA for antigen-specific antibody.* Whole blood was collected from immunized animals by heart puncture at the end of the study. The SST tubes were centrifuged at 800 rpm for 20 minutes. Sera were aliquoted and stored at -20°C until assayed. PVC plates (polychlorinated biphenyl plates, Falcon, Oxnard, California) were coated with native p24 diluted in PBS at 1µg/ml and stored at 4°C overnight. Plates were blocked by adding 200µl per well of 4% BSA in PBS for 1 hour. Sera were diluted in 1% BSA in PBS at 1:100 followed by four-fold serial dilution. 100µl of diluted sera were added in duplicate and incubated at room temperature for 2 hours. Plates were washed with 0.05% Tween 20 in PBS three times and blotted dry. The detecting secondary antibodies (goat anti-rat IgG biotin, goat anti-rat IgG1 biotin or goat anti-rat IgG2a biotin, Zymed, San Francisco, California) were diluted in 1% BSA in PBS. 100µl of diluted secondary antibody was added to each well and incubated at room temperature for another hour. After washing excess secondary antibody, strep-avidin-biotin-HRP (Pierce, Rockford, Illinois) were added at 50µl per well and incubated for 30 minutes. Plates were washed with 0.05% Tween 20 in PBS three times. ABTS substrate (KPL, Gaithersburg, Maryland) was added until a bluish-green color developed. The reaction was stopped by the addition of 1% SDS and the plate was read at absorbance 405 nm.

The antibody response reported as 50% antibody titer was the reciprocal of the dilution equal to 50% of the maximum binding (highest optical reading) for every given sample. The absorbance value (OD @ 405 nm) was plotted against antibody dilution in a log scale, yielding a sigmoidal dose response curve. 50% of the maximum binding was calculated by multiplying the highest

OD by 0.5. The 50% value was located on the curve and the corresponding x-axis value was reported as the antibody dilution.

*ELISA Assay for Cytokine and Chemokine Analysis.* The draining lymph nodes (superficial inguinal and popliteal) were isolated from immunized animals two weeks after the boost. Single cell suspensions from these lymph nodes were prepared by mechanical dissociation using sterile 70  $\mu\text{m}$  mesh screen. T cells were purified from lymph node cells by the panning method. Briefly, petri dishes (100 x 15mm) were pre-coated with 20 $\mu\text{g}/\text{ml}$  of rabbit anti-rat IgG (Rockland, San Francisco, California) for 45 minutes at room temperature. The petri dishes were washed twice with ice cold PBS and once with ice cold 2% human AB serum in PBS.  $1 \times 10^7$  lymph node cells were added to pre-washed plates and incubated at 4°C for 90 minutes. The non-adherent cells (enriched T cells) were then collected and transferred into sterile 50-ml conical tubes. The plates were washed twice and combined with the non-adherent cells. The cells were then centrifuged and cell pellets resuspended in complete media at  $4 \times 10^6$  cells/ml (5% human AB serum in RPMI 1640, with 25 mM hepes, 2mM L-glutamine, 100  $\mu\text{g}$  streptomycin and  $5 \times 10^{-6}\text{M}$   $\beta$ -mercaptoethanol).

Gamma-irradiated thymocytes from a naive Lewis rat were used as antigen presenting cells.  $2 \times 10^5$  enriched T cells and  $5 \times 10^5$  thymocytes were added to each well of a 96-round bottom plate. The HIV-1 antigen and native p24 were diluted in complete media at 10 $\mu\text{g}/\text{ml}$  while con A was diluted to 5 $\mu\text{g}/\text{ml}$ . 100 $\mu\text{l}$  of each antigen or T cell mitogen were added in triplicates. The plates were incubated at 5%  $\text{CO}_2$ , 37°C for 72 hours. Supernatants were harvested and stored at -70°C until assayed. The samples

were assayed for IL-4, IFN- $\gamma$  and RANTES using commercially available kits (Biosource, Camarillo, California) specific for rat cytokines and chemokines.

*Statistical methods.* The Mann-Whitney U nonparametric  
5 statistic was utilized to compare groups. All *p* values are two tailed.

### Results

As shown in Figure 1A, administration of envelope-depleted HIV-1 in combination with IFA and ISS  
10 (ODN 1826) was a more potent inducer of both HIV-1 antigen-stimulated and p24 antigen-stimulated IFN- $\gamma$  production than HIV-1 in CFA ( $p=.002$ ), HIV-1 in IFA, or HIV-1 in ISS ( $p=.02$ ). Increased production of unstimulated IFN- $\gamma$  (control) was also observed following  
15 administration of envelope-depleted HIV-1 in combination with IFA and ISS. Unexpectedly, administration of HIV-1 in combination with IFA and ISS resulted in IFN- $\gamma$  production that was several times greater than the additive effects of HIV-1 in IFA alone or HIV-1 in ISS  
20 alone. Of note, the level of cytokine secreted after HIV-1 stimulation was higher than after p24 stimulation, due to the presence of multiple T cell epitopes in the whole HIV-1 antigen.

Complete Freund's Adjuvant (CFA) is currently  
25 the most potent adjuvant known for stimulating cell-mediated immune responses. However, CFA is not an appropriate adjuvant for use in humans because of safety issues. As shown in Figure 1A, HIV in CFA induced unstimulated and HIV-stimulated IFN- $\gamma$  production more  
30 effectively than HIV in IFA alone or HIV in ISS alone, but not as well as HIV in the combination of IFA and ISS.

Thus, the discovery of the superior effects of the combination of ISS and IFA for use in an HIV immunogenic composition provides for safe and effective vaccines for human therapy.

5                   To examine the dose-related immune response to IFN- $\gamma$ , Lewis rats were immunized with the inactivated gp120-depleted HIV-1 antigen emulsified in IFA containing different concentrations of CpG ODN 1826 (50, 25 and 5  $\mu$ g per rat). The highest production of antigen-stimulated  
10 IFN- $\gamma$  was obtained using 50  $\mu$ g of CpG ODN 1826, as shown in Figure 1B.

                  To examine whether CpG ODN could also boost the antibody response to an HIV-1 antigen, sera were assayed  
15 for total IgG and Th2 isotype (IgG1 and IgG2a) antibody responses to p24 antigen. As shown in Figure 2A, anti-p24 total IgG responses were strongly enhanced and comparable in both the HIV in CFA and HIV in IFA/ISS groups of animals. Administration of HIV-1 in  
20 combination with IFA and ISS resulted in total p24 antibody production that was greater than the additive effects of HIV-1 in IFA alone or ISS alone, and almost as great as HIV-1 in CFA. The IgG1 and IgG2a responses were comparable among animals immunized with HIV-1 antigen in  
25 CFA, IFA or IFA/ISS. As shown in Figure 2B, the antibody response was dependent on the dose of ISS.

                  Production of the  $\beta$ -chemokine RANTES in response to immunization was then examined. As shown in Figure 3A, both unstimulated and antigen-stimulated cells  
30 from the HIV/IFA/ISS group showed enhanced production of RANTES, to a level comparable with the HIV in CFA group, and significantly higher than the HIV/IFA group ( $p=.002$ ) or HIV/ISS group ( $p=.02$ ). Unexpectedly, administration

of HIV-1 in combination with IFA and ISS resulted in both unstimulated and antigen-stimulated RANTES production that was greater than the additive effects of HIV-1 in IFA alone or HIV-1 in ISS alone. As shown in Figure 3B, both unstimulated and antigen-stimulated RANTES production was dependent on the dose of ISS.

In none of the groups was production observed of antigen-induced IL-4, a Th2 type cytokine. The control sequence (1745) did not stimulate IFN- $\gamma$ , RANTES, or p24 antibody.

Cytokine and chemokine production was compared with compositions containing two oligonucleotides containing different immunostimulatory sequences. As shown in Figure 4A, immunogenic compositions containing HIV-1 antigen and IFA with either ODN 1826 (SEQ ID NO:1) or ODN Oct (SEQ ID NO:2) induced antigen-stimulated IFN- $\gamma$  production to a greater extent than compositions containing HIV-1 antigen and IFA, or HIV-1 antigen and CFA. Furthermore, as shown in Figure 4B, immunogenic compositions containing HIV-1 antigen and IFA with either ODN Oct or ODN 1826 induced unstimulated and antigen-stimulated RANTES production to a greater extent than compositions containing HIV-1 antigen and IFA, or HIV-1 antigen and CFA.

Thus, the immunogenic compositions of the invention can be used to enhance  $\beta$ -chemokine production in an individual. Because of the strong correlation between  $\beta$ -chemokine levels and protection from HIV infection and disease progression, the compositions of the invention will be more effective than other described compositions for inhibiting AIDS.

EXAMPLE IIElicitation of CD4 and CD8 immune responses  
by HIV immunogenic compositions

This example shows the induction of potent CD4  
5 and CD8 HIV-specific Th1 type immune responses following  
immunization with an immunogenic composition containing  
an HIV antigen, a nucleic acid containing an  
immunostimulatory sequence and an adjuvant. Antigen-  
specific responses by CD8+, cytotoxic T lymphocytes are  
10 an important factor in preventing initial HIV infection  
and disease progression. Thus, this example provides  
further evidence that the immunogenic compositions of the  
invention are effective prophylactic and therapeutic  
vaccines.

15 Materials and Methods

HIV antigen, ISS (ODN 1826) and IFA were  
prepared essentially as described in Example I. Lewis  
rats were immunized essentially as described in Example  
I, and sacrificed at day 28 for ELISPOT and p24 antibody  
20 analysis. p24 antibody analysis was performed  
essentially as described in Example I.

*ELISPOT for gamma-interferon from bulk and purified T  
cell populations.* Single cell suspensions were prepared  
25 from spleens of the immunized rats by mincing and  
pressing through a sterile fine mesh nylon screen in RPMI  
1640 (Hyclone, Logan, Utah). The splenocytes were  
purified by ficoll gradient centrifugation. CD4 and CD8  
cells were isolated by magnetic bead depletion.  $2 \times 10^7$   
30 cells were stained with 5µg of either mouse anti-rat CD4  
(clone: OX-35, Pharmingen, San Diego, California) or

mouse anti-rat CD8 (clone: OX-8, Pharmingen, San Diego, California). Cells were incubated on ice for 30 minutes and washed with ice cold 2% Human AB serum in PBS. Pre-washed Dynabeads (DYNAL, Oslo, Norway) coated with goat anti-mouse IgG were added to the cell suspension and incubated at 4°C for 20 minutes with constant mixing.

Purified CD4, CD8 and non-depleted splenocytes were resuspended in complete media (5% inactivated Human AB serum in RPMI 1640, Pen-strep, L-glutamine and  $\beta$ -ME) at  $5 \times 10^6$  cells/ml and used for ELISPOT assay to enumerate the individual IFN- $\gamma$  secreting cells. Briefly, 96 well nitrocellulose bottom microtiter plates (Millipore Co., Bedford, U.K.) were coated with 400 ngs per well of mouse anti-rat IFN- $\gamma$  (clone: DB-1, Biosource, Camarillo, California). After overnight incubation at 4°C, plates were washed with sterile PBS and blocked with 5% human AB serum in RPMI 1640 containing pen-strep, L-glutamine and  $\beta$ -ME) for 1 hour at room temperature. Plates were washed with sterile PBS and  $5 \times 10^5$  per well of splenocytes (purified CD4, purified CD8 or non-depleted) were added in triplicate and incubated overnight at 37°C and 5% CO<sub>2</sub>. Cells were cultured with media, OVA (Chicken Egg Ovalbumin, Sigma-Aldrich, St. Louis, Missouri), native p24 or gp120-depleted HIV-1 antigen. CD4 purified and CD8 purified splenocytes were assayed in complete media containing 20 units/ml of recombinant rat IL-2 (Pharmingen, San Diego, CA).

After washing unbound cells, 400 ng per well of the polyclonal rabbit anti-rat IFN- $\gamma$  were added and incubated at room temperature for 2 hours, then washed and stained with goat anti-rabbit IgG biotin (Zymed, San Francisco, California). After extensive washes with sterile PBS, avidin alkaline phosphatase complex (Sigma-



Aldrich, St. Louis, MO) was added and incubated for another hour at room temperature. The spots were developed by adding chromogenic alkaline phosphate substrate (Sigma, St. Louis, MO) and the IFN- $\gamma$  cells were  
5 counted using a dissection microscope (X 40) with a highlight 3000 light source (Olympus, Lake Success, NY).

*Statistical Methods.* The Mann-Whitney U nonparametric statistic was utilized to compare groups. The Spearman rank correlation was performed to examine relationships  
10 between CD4 and CD8 gamma interferon production. All p values are two tailed.

### Results

The production of IFN- $\gamma$  by non-depleted splenocytes, and by purified CD4+ or purified CD8+  
15 populations, was examined. IFN- $\gamma$  production by CD4+ cells is a characteristic Th1 immune response, whereas IFN- $\gamma$  production by CD8+ cells is a correlate of cytotoxic T lymphocyte (CTL) cytolytic activity.

The frequency of IFN- $\gamma$  producing cells  
20 increased with dose of ISS in non-depleted splenocytes in response to either whole-killed, gp120-depleted HIV (the immunizing antigen) or purified p24 antigen (see Figure 5A). The highest frequency of cytokine producing cells was observed with the combination of 100  $\mu$ g of ISS  
25 with HIV-1 in IFA, for both HIV-1 and p24 antigen stimulated cells ( $p=0.03$  when compared the HIV in IFA group).

The purified CD4+ T cell population also  
30 exhibited a dose-dependent increase in the frequency of cells expressing IFN- $\gamma$  in response to HIV and p24

antigens, with the greatest frequency being at the 100 µg dose of ISS when combined with HIV-1 in IFA ( $p=0.03$  when compared the HIV in IFA group) (see Figure 5B). Furthermore, the purified CD8+ population also exhibited  
5 a dose-dependent increase in the frequency of cells expressing IFN- $\gamma$  in response to HIV and p24 antigens, with the greatest frequency being at the 100 µg dose of ISS when combined with HIV-1 in IFA ( $p=0.03$  when compared the HIV in IFA group) (see Figure 5C). None of the  
10 animals produced IFN- $\gamma$  secreting cells when stimulated with OVA, an irrelevant protein antigen.

Of note, the frequency of IFN- $\gamma$  producing CD8+ T cells was generally lower than the frequency of CD4+ T cells expressing IFN- $\gamma$ . There was a strong correlation  
15 between the generation of IFN- $\gamma$  between CD4+ T cells and CD8+ T cells with both HIV antigen stimulation ( $r=0.80$ ,  $p=0.002$ ) and for p24 antigen stimulation ( $r=0.79$ ,  $p=0.003$ ).

The results shown in Figures 5A, B and C thus  
20 demonstrate that the immunogenic compositions of the invention elicit Th1 and cytotoxic T lymphocyte responses, both of which are correlated with protection from initial HIV infection and progression to AIDS.

Finally, total IgG, IgG1 and IgG2b specific for  
25 p24 was examined. As shown in Figure 6A, the addition of ISS at all doses to HIV in IFA increased anti-p24 antibody response (total IgG) compared to HIV in IFA, although a dose response was not evident. Specifically, the addition of ISS to HIV in IFA favored the production  
30 of IgG2b antibody (a Th1 type response) compared to HIV in IFA, which induced only IgG1 subtype antibody (a Th2 type response), as shown in Figure 6B.

In summary, the data in this Example show that an immunogenic composition containing an HIV antigen, an ISS and an adjuvant can be used to generate potent HIV-specific CD4 and CD8 HIV-specific immune responses. The induction of CD4 T helper cells may be pivotal for generation of CD8 effector cells. CD8 T cells can serve as effectors against HIV virus by several mechanisms, including direct cytolytic (CTL) activity, as well as through the release of antiviral suppressive factors, such as  $\beta$ -chemokines and other less well-characterized factors. These results contrast with results reported by Deml et al., supra (1999), who showed that a combination of HIV envelope gp160 antigen, an ISS and an adjuvant did not induce HIV-specific CTL activity. Accordingly, the compositions described herein are superior to other described compositions for use as HIV vaccines.

### EXAMPLE III

#### Comparison of immune responses elicited by different immunogenic compositions and immunization schedules

This example shows that a nucleic acid containing an ISS is more effective in eliciting protective immune responses, including RANTES production and HIV-specific IgG2b antibody production, when administered simultaneously with an HIV antigen and an adjuvant than when used to prime the mammal one week prior to administration of the antigen and adjuvant. This example also shows that a composition containing an HIV antigen, an ISS and an adjuvant promotes antigen-dependent lymphocyte proliferation more effectively than a composition containing only HIV and IFA.

Materials and methods

HIV antigen, ISS (ODN 1826) and IFA were prepared essentially as described in Example I. Lewis rats (three per group) were immunized at day 7 and, where indicated, primed at day 0, with the compositions shown in Table 2.

Table 2

10	<u>Group</u>	<u>Day 0</u>	<u>Day 7</u>
	A	ISS	HIV-1
	B		HIV-1
	C	ISS	HIV-1/IFA
	D		HIV-1/IFA
	E		HIV-1/IFA/ISS

15               Animals were sacrificed at day 21 for cytokine, chemokine and antibody analysis, essentially as described in Example I, as well as for analysis of lymphocyte proliferation.

*Lymphocyte proliferation assay.* Single cell suspensions were prepared from the draining lymph nodes of immunized animals. B cells were depleted from the lymph node cells by panning. Briefly, lymph node cells were incubated with anti-rat IgG pre-coated petri dishes for 90 minutes. The non-adherent cells (enriched T cells) were collected and resuspended in complete tissue culture media at  $4 \times 10^6$  cells/ml. The enriched T cells were cultured with p24 or HIV-1 antigen in the presence of  $\gamma$ -irradiated thymocytes at 37°C, 5% CO<sub>2</sub> for 40-48 hours. Samples were pulsed with tritiated thymidine and incubated for another 16 hours.

25               Cells were harvested and tritiated thymidine

30

incorporation was counted using a  $\beta$ -scintillation counter.

### Results

5           As shown in Figure 7A, T cells from animals primed with ISS and subsequently boosted with HIV-1 in IFA (Group C), animals immunized with HIV-1 in IFA, and animals immunized with a combination of HIV-1, IFA and ISS (Group E), exhibited increased IFN- $\gamma$  production in  
10 response to whole-killed, gp120-depleted HIV (the immunizing antigen) and a lesser increase in IFN- $\gamma$  production in response to purified p24 antigen.

          However, as shown in Figure 7B, only T cells from animals immunized with a combination of HIV-1, IFA  
15 and ISS (Group E) showed high levels of either non-stimulated (media), or HIV-stimulated RANTES production. RANTES production from animals of Group E was several fold higher than from animals primed with ISS, then boosted one week later with HIV-1 in IFA (Group C).

20           Serum levels of total IgG, IgG1 and IgG2b specific for p24 antigen were also examined. As shown in Figure 7C, animals immunized with a combination of HIV-1, IFA and ISS (Group E) showed the highest levels of total IgG. Unexpectedly, whereas animals not receiving ISS  
25 (Group D) and animals primed with ISS (Group C) produced primarily IgG1 (Th2-type) antibodies, animals immunized with a combination of HIV-1, IFA and ISS (Group E) produced primarily IgG2b (Th1-type) antibodies (see Figure 7D).

30           T cell proliferative responses to p24 antigen and gp120-depleted HIV were also measured. As shown in

Figure 7E, T cells from animals immunized with a combination of HIV-1, IFA and ISS (Group E) proliferated more strongly in response to either gp120-depleted HIV or p24 antigen than did T cells from animals primed with ISS then administered HIV-1 in IFA one week later (Group C),  
5 or from animals administered only HIV-1 in IFA (Group D).

Thus, the immunogenic compositions of the invention effectively elicit HIV-specific Th1 cytokine (IFN- $\gamma$ ) and humoral responses (IgG2 antibodies), and  
10 enhance both non-specific and HIV-specific  $\beta$ -chemokine production. These responses to the immunogenic compositions correlate with strong HIV-specific T lymphocyte proliferative responses.

#### EXAMPLE IV

##### 15 Immunization of a primate with an HIV immunogenic composition

This example shows that immunogenic compositions containing an HIV antigen, an isolated nucleic acid molecule containing an ISS and an adjuvant  
20 are effective in enhancing HIV-specific immune responses in primates.

Three baboon fetuses were injected *in utero* with an immunogenic composition containing gp120-depleted HIV-1 (100  $\mu$ g total protein, equivalent to 10 p24 units)  
25 in IFA with 500  $\mu$ g of the ISS designated ODN 2006. The sequence of ODN 2006 is 5'-TCGTCGCTGTTGTCGTTTCTT-3' (SEQ ID NO:4). Four weeks later, the fetuses were boosted using the same regimen.

Peripheral blood mononuclear cells from the neonatal baboons were collected, and proliferative responses to p24 and HIV-1 antigen were assayed. As shown in Table 3, in all three animals, the HIV-1 stimulation index, which is the ratio of T cell proliferation ( $^3\text{H}$  incorporation) in response to antigen to T cell proliferation without antigen, was indicative of a strong immune response (i.e. stimulation index  $>3$ ). Two baboon fetuses injected *in utero* and boosted as neonates showed similar results.

Table 3

Baboon	HIV-1 Stimulation Index
6533	13.3
5924	5.87
6683	15.1

Production of HIV-specific antibodies, cytokines and  $\beta$ -chemokines are also measured in the same baboons. These results show that the types of immune responses elicited by the immunogenic compositions described in Examples I-III, above, for rodents, are also elicited in primates.

These results demonstrate that the HIV immunogenic compositions and methods of the invention are effective in primates in stimulating HIV-specific immune responses. Furthermore, these results demonstrate that fetuses and infants are able to elicit strong HIV immune responses to the immunogenic compositions of the invention, indicating that these compositions will be useful for preventing maternal transmission of HIV and as pediatric vaccines.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to  
5 more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific  
10 experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.



What is claimed is:

1. An immunogenic composition, comprising:
  - (a) a whole-killed HIV virus devoid of outer envelope protein gp120;
  - 5 (b) an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS); and
  - (c) an adjuvant,which enhances  $\beta$ -chemokine levels in a mammal.
- 10 2. The immunogenic composition of claim 1, wherein said HIV virus is HIV-1.
3. The immunogenic composition of claim 1, wherein said HIV virus is an HZ321 strain virus.
4. The immunogenic composition of claim 1,  
15 wherein said isolated nucleic acid molecule is an oligodeoxynucleotide.
5. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises two or more CpG sequences.
- 20 6. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises at least one sequence consisting of 5'-Cytosine, Guanine, Pyrimidine, Pyrimidine-3'.
7. The immunogenic composition of claim 1,  
25 wherein said isolated nucleic acid molecule comprises at least one sequence consisting of 5'-CGTT-3'.

8. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises at least one sequence consisting of 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine-3'

5 9. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises at least one sequence consisting of 5'-GACGTT-3'.

10 10. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises at least one sequence consisting of 5'-TCCATGACGTTCTGACGTT-3' (SEQ ID NO:1).

11. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises at least one sequence consisting of 5'-AACGTTCG-3'.

15 12. The immunogenic composition of claim 1, wherein said isolated nucleic acid molecule comprises a phosphorothioate backbone.

13. The immunogenic composition of claim 1, wherein said HIV virus is conjugated to said nucleic acid  
20 molecule.

14. The immunogenic composition of claim 1, wherein said adjuvant is suitable for use in humans.

15. The immunogenic composition of claim 1,  
25 wherein said adjuvant comprises incomplete Freund's adjuvant (IFA).

16. The immunogenic composition of claim 1,  
wherein said adjuvant comprises mycobacterium cell wall  
components and monophosphoryl lipid A.

17. The immunogenic composition of claim 1,  
5 wherein said adjuvant comprises alum.

18. The immunogenic composition of claim 1,  
wherein said enhanced  $\beta$ -chemokine production is non-  
specific  $\beta$ -chemokine production.

19. The immunogenic composition of claim 1,  
10 wherein said enhanced  $\beta$ -chemokine production is HIV-  
specific  $\beta$ -chemokine production.

20. The immunogenic composition of claim 1,  
wherein said  $\beta$ -chemokine is RANTES.

21. The immunogenic composition of claim 1,  
15 said composition further capable of enhancing HIV-  
specific IgG2b antibody production in a mammal.

22. The immunogenic composition of claim 1,  
said composition further capable of enhancing an HIV-  
specific cytotoxic T lymphocyte (CTL) response in a  
20 mammal.

23. A kit, comprising:

- (a) a whole-killed HIV virus devoid of  
outer envelope protein gp120;
- 25 (b) an isolated nucleic acid molecule  
containing an immunostimulatory  
sequence (ISS); and
- (c) an adjuvant,

said kit components, when combined, producing the  
30 immunogenic composition of claim 1.

24. A method of making the immunogenic composition of claim 1, comprising combining:

- (a) a whole-killed HIV virus devoid of outer envelope protein gp120;
- 5 (b) an isolated nucleic acid molecule containing an immunostimulatory sequence (ISS); and
- (c) an adjuvant.

25. The method of claim 24, wherein said  
10 combining is *ex vivo*.

26. The method of claim 24, wherein said combining is *in vivo*.

27. A method of immunizing a mammal, comprising enhancing  $\beta$ -chemokine production in the mammal  
15 by administering to the mammal the immunogenic composition of claim 1.

28. A method of inhibiting AIDS, comprising enhancing  $\beta$ -chemokine production in a mammal by administering to the mammal the immunogenic composition  
20 of claim 1.

29. The method of claim 27 or claim 28, wherein said mammal is a primate.

30. The method of claim 29, wherein said primate is an infant.

25 31. The method of claim 29, wherein said primate is pregnant.

32. The method of claim 29, wherein said primate is a human.

33. The method of claim 32, wherein said human is HIV seronegative.

5           34. The method of claim 32, wherein said human is HIV seropositive.

35. The method of claim 27, wherein said mammal is a rodent.

36. The method of claim 27 or claim 28,  
10 wherein said composition is administered to said mammal two or more times.

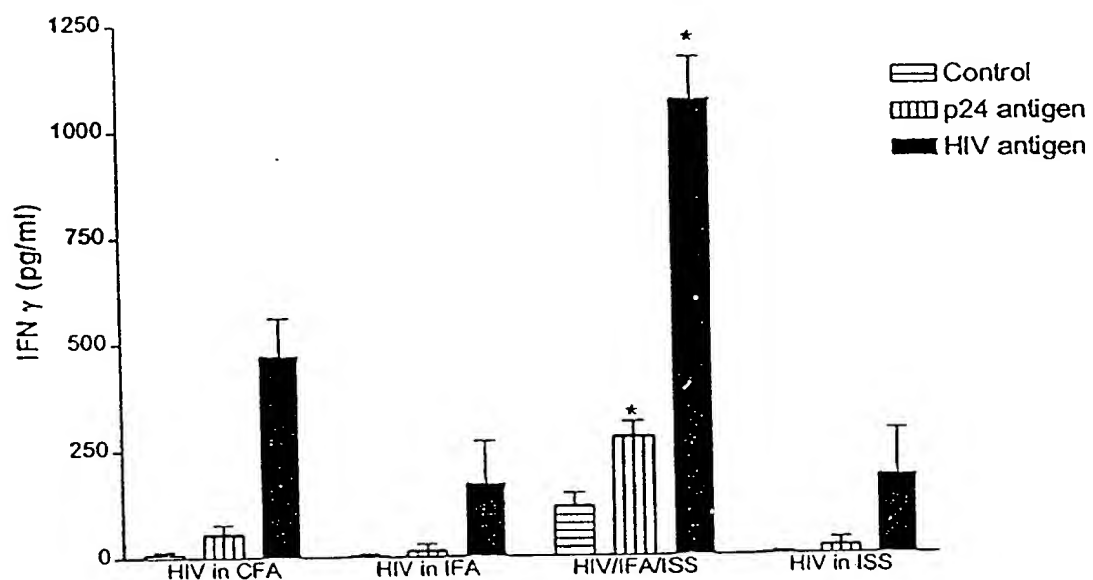


Figure 1A

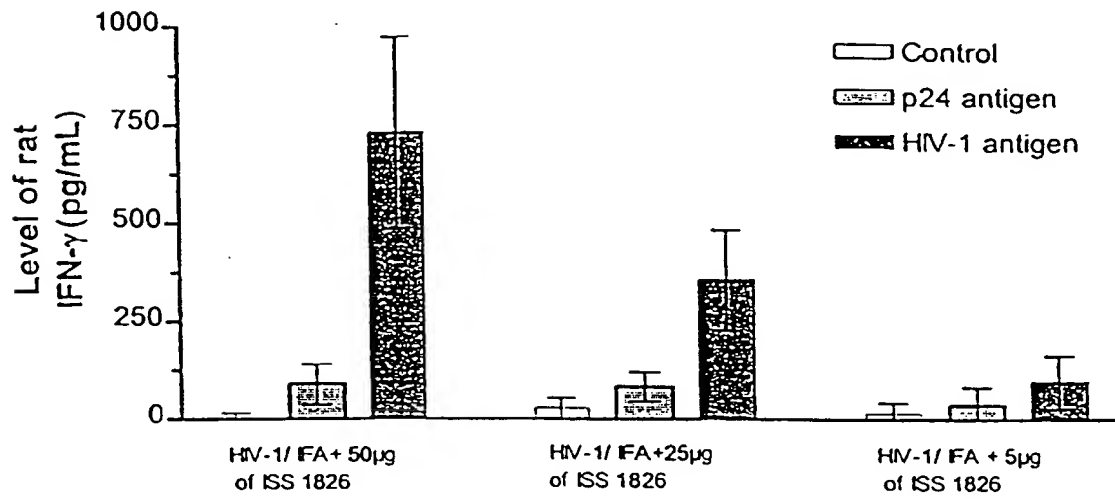


Figure 1B

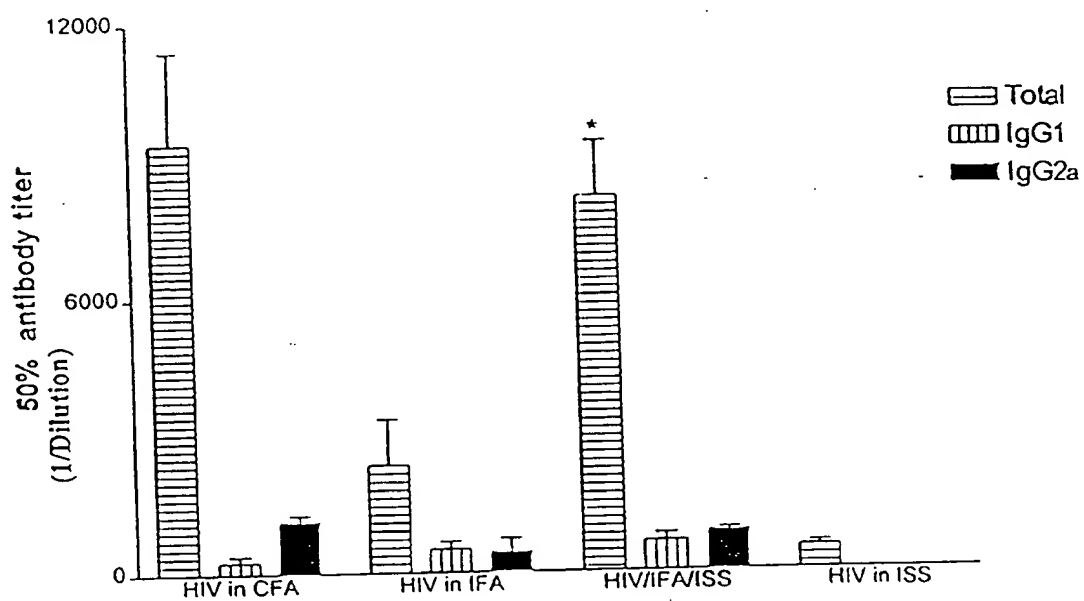


Figure 2A



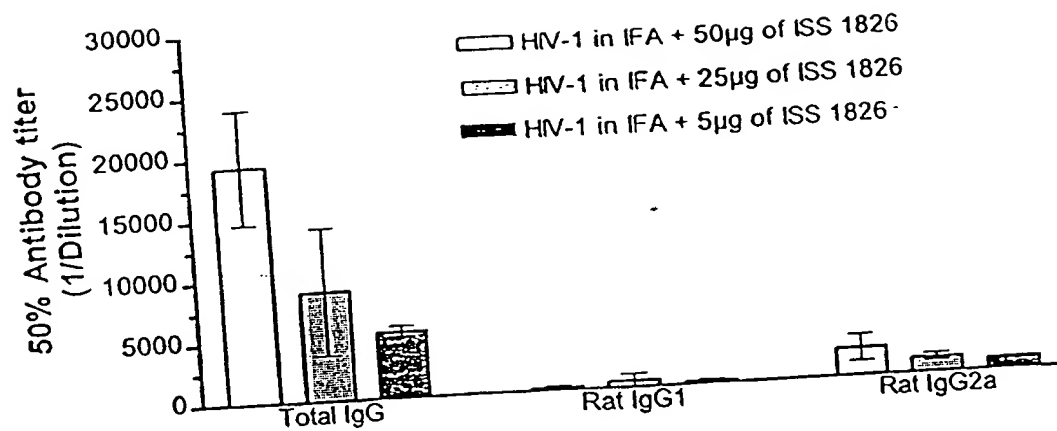


Figure 2B

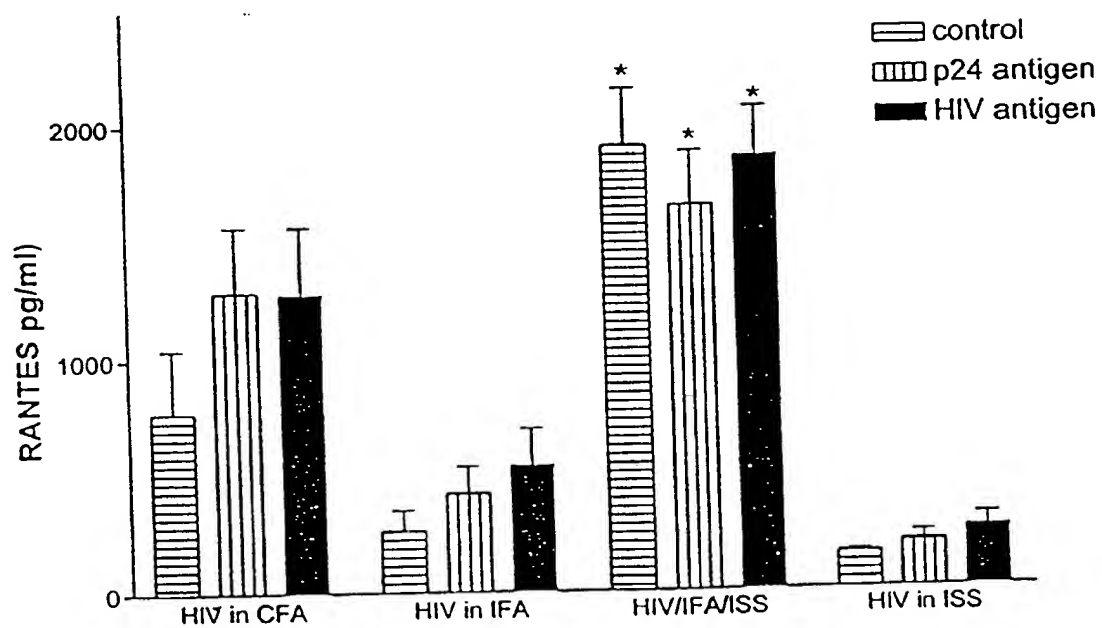


Figure 3A

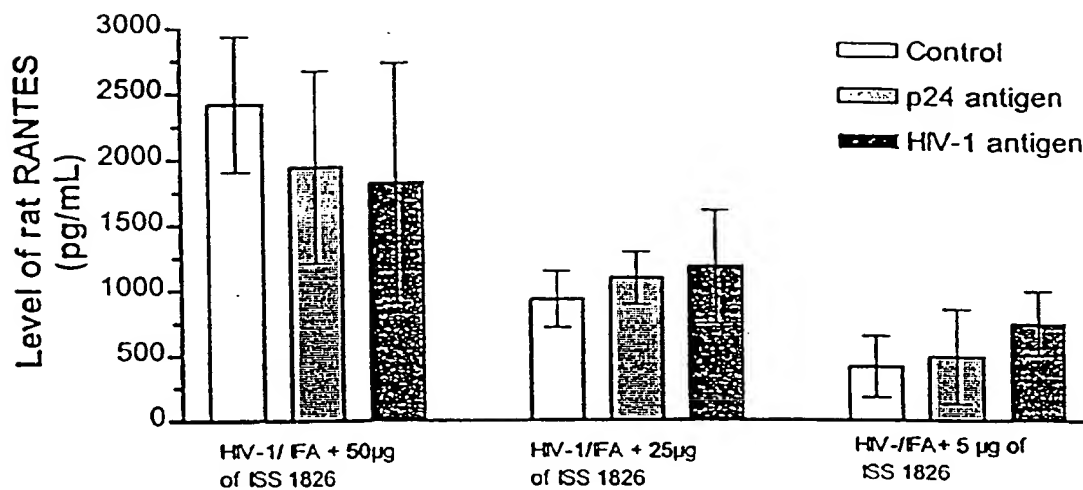


Figure 3B

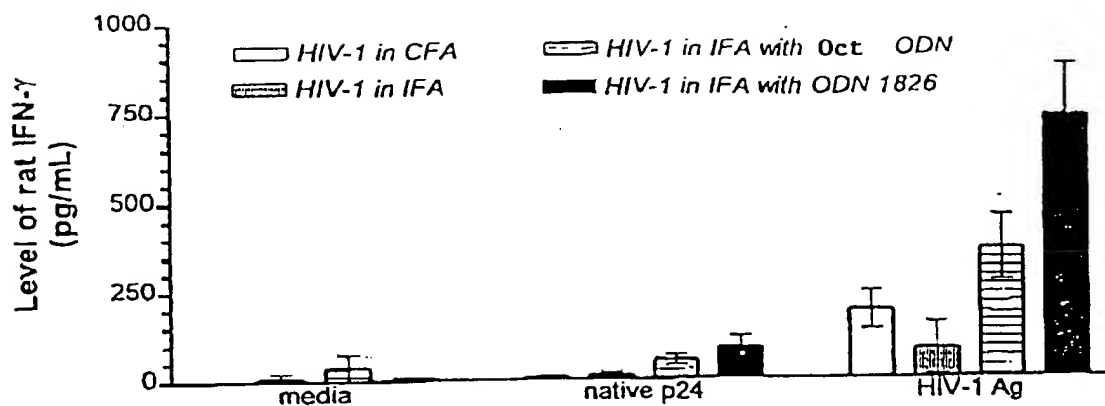


Figure 4A

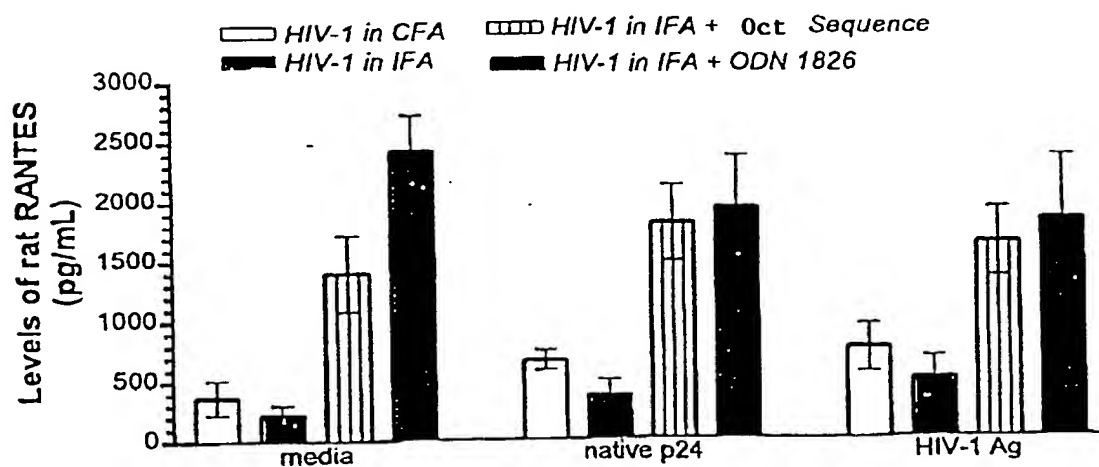


Figure 4B

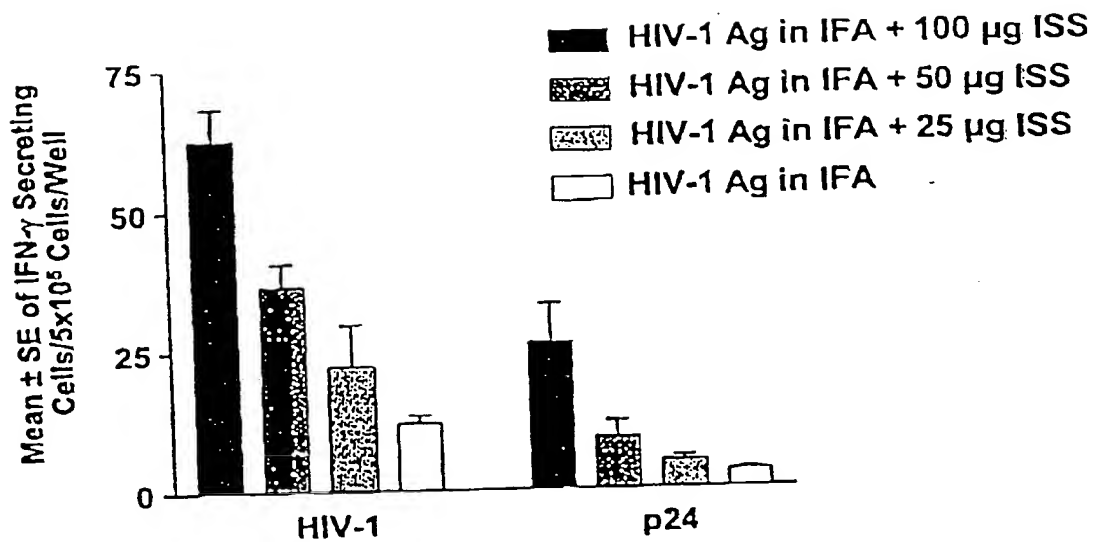


Figure 5A

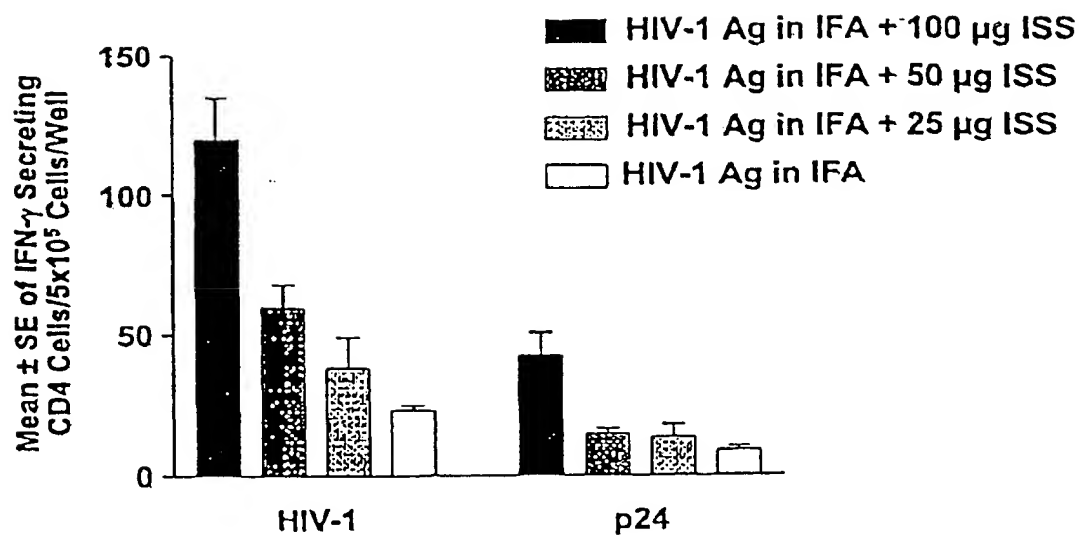


Figure 5B

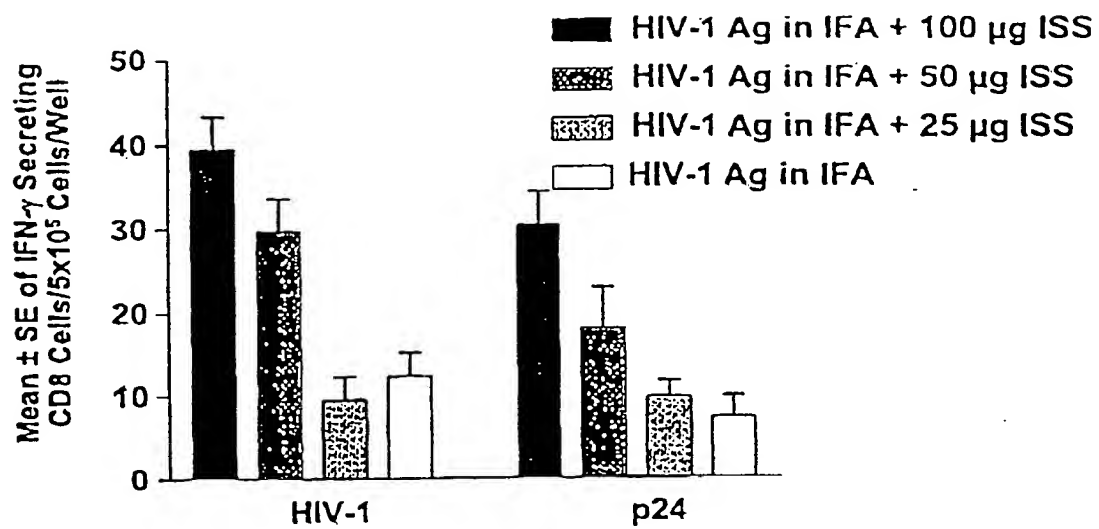


Figure 5C

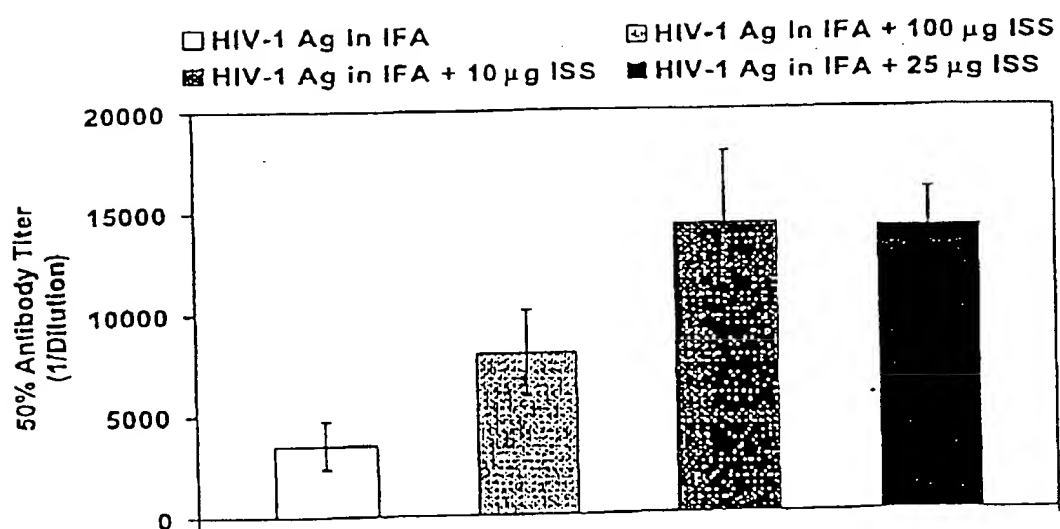


Figure 6A



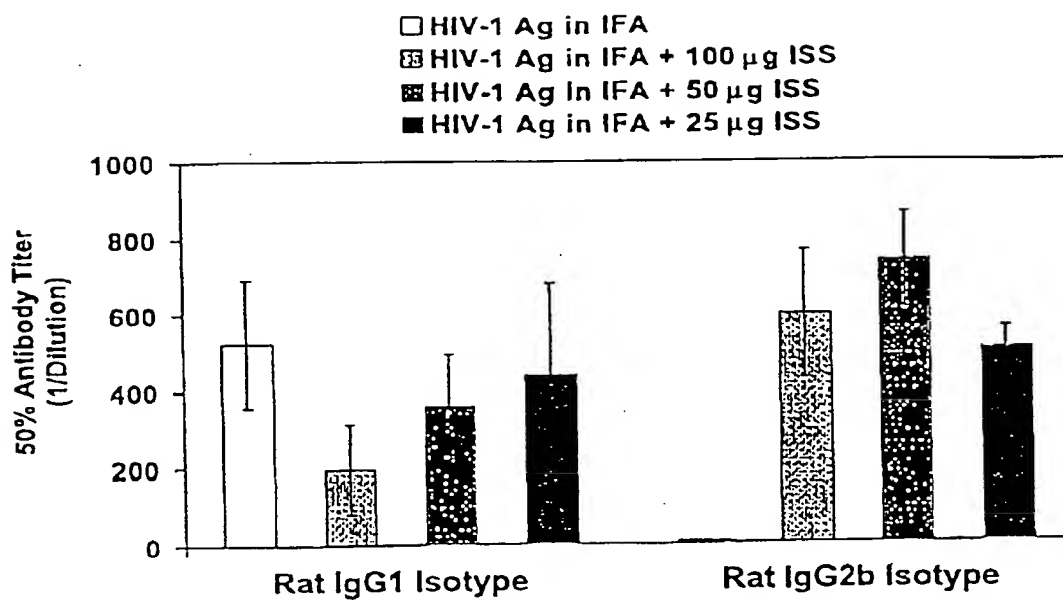


Figure 6B

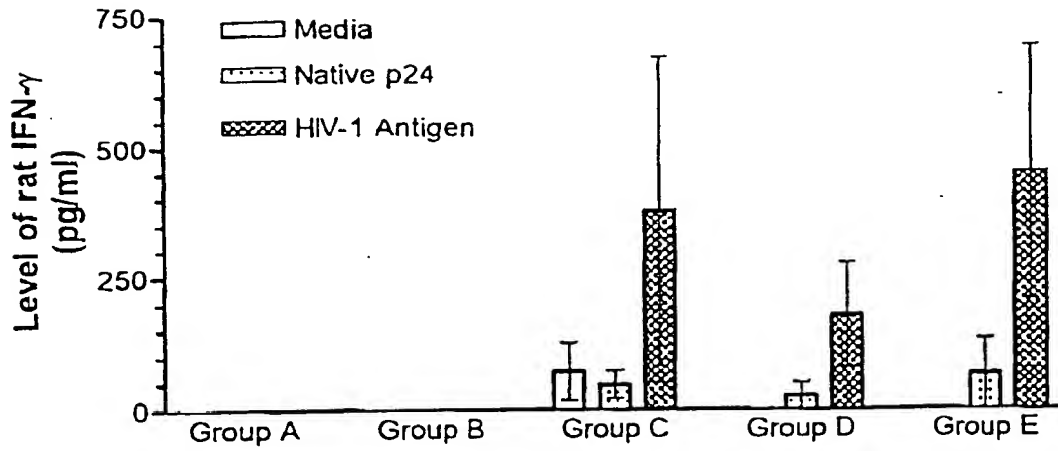


Figure 7A

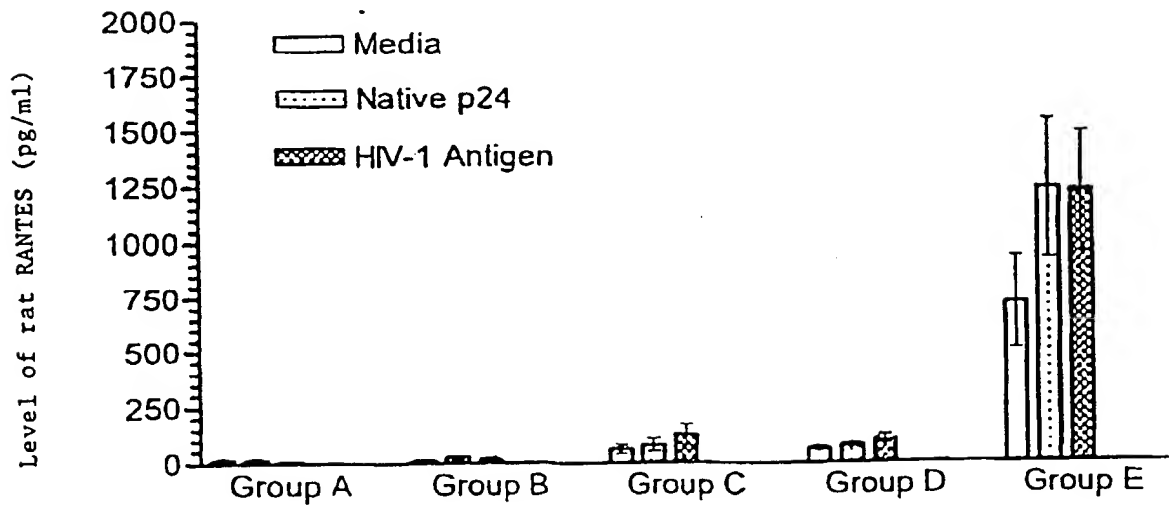


Figure 7B

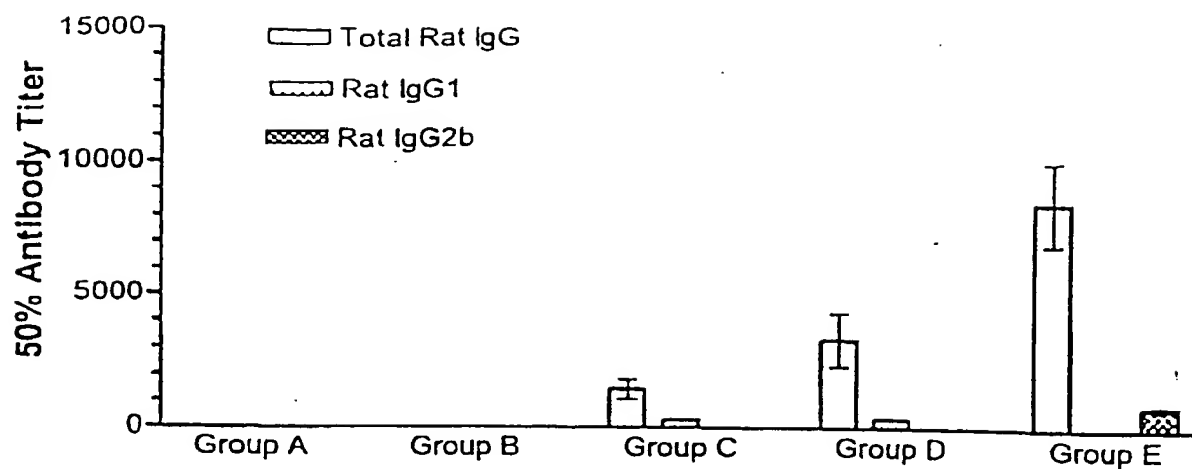


Figure 7C

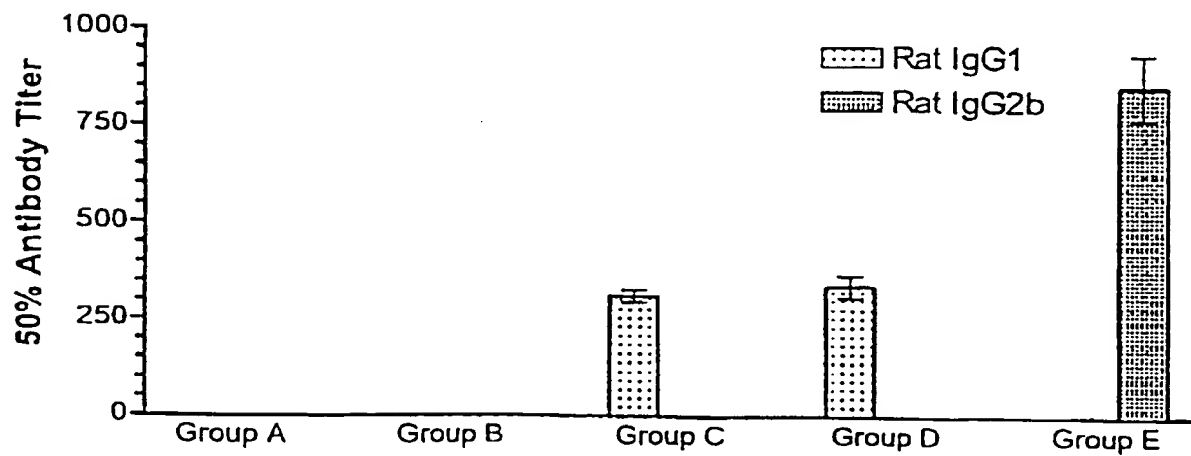


Figure 7D

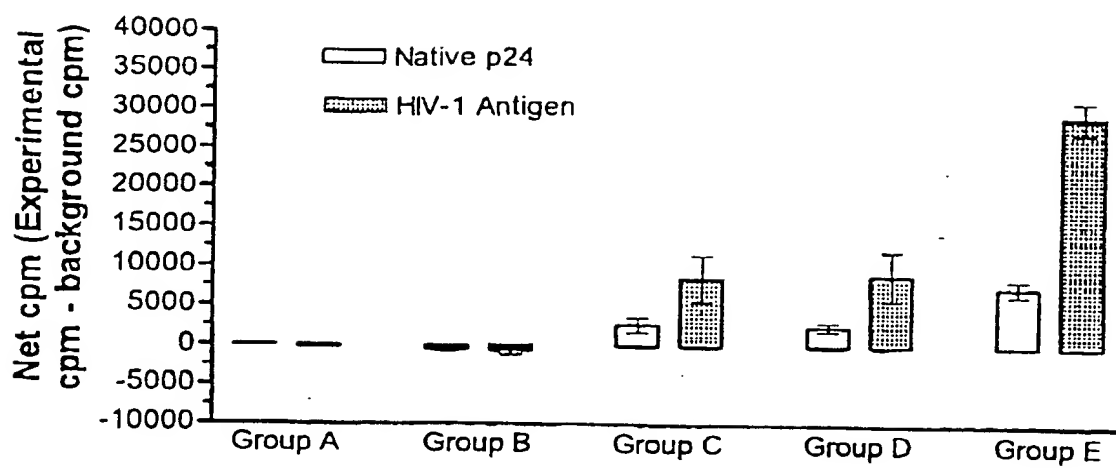


Figure 7E